THE
BIOLOGICAL BULLETIN

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ON THE GENUS ANCISTRUMA STRAND (ANCISTRUM MAUPAS)

I. The Structure and Division of A. Mytili Quenn. and A. Isseli Kahl

GEORGE W. KIDDER
College of the City of New York

Introduction

In 1867 Quennerstedt described a small ciliate from the mantle cavity of *Mytilus* under the name of *Opalina mytili*. Kent (1882) placed this form in the genus *Anoplophrya* and retained the species name given by Quennerstedt. Maupas (1883) redescribed this ciliate, calling attention to the presence of a mouth, which had been overlooked by Quennerstedt and Kent. He created the genus *Ancistrum* for the type species of Quennerstedt and described a second species, *A. veneris gallinæ*. Strand (1926), in reviewing the literature, finds the name *Ancistrum* preoccupied and therefore invalid. He proposes the name *Ancistruma* to replace the commonly employed one of *Ancistrum*.

It is the purpose of this paper to report some observations on two species of the genus *Ancistruma*, namely, *A. mytili* Quennerstedt and *A. isseli* Kahl.

In his excellent monograph on the Ancistridæ of the Gulf of Naples, Issel (1903) describes in detail an *Ancistrum* from the solitary mussel *Modiola barbata*. Although his findings were not entirely in accord with previous descriptions he believed this form to be identical to *A. mytili* and placed it in that species. Kahl (1931), however, called this form *Ancistruma isseli*. I am entirely in agreement with Kahl on this point, as will be brought out in the following pages.

This investigation was carried on at the Marine Biological Laboratory at Woods Hole, Massachusetts, and the Zoölogical Laboratories of Columbia University in New York City. I am indebted to the faculty of Columbia University for their generosity in providing me with research facilities. I am especially indebted to Professor Gary N. Calkins

for kindly advice and criticisms and to Ruth R. Kidder for technical assistance.

Material

Ancistruma mytili is found in great numbers in the mantle cavity of the salt water mussel *Mytilus edulis*. These mussels were collected from the pilings of the wharf at the Bureau of Fisheries at Woods Hole and from the rock breakwaters at Brighton Beach, Manhattan Beach, and Port Washington, Long Island. *A. isseli* is equally abundant in the mantle cavity of the solitary mussel *Modiola modiolus*. This mussel may be found half buried in the muddy flats at low tide. The *Modiola* used in this work were collected just north of the Coast Guard station at Woods Hole. Additional mussels of both genera were taken from Pelham Bay in New York City.

Technique

The mussels were brought into the laboratory and placed in large aquaria supplied with running sea water (Woods Hole) or a jet of air (New York). In this situation the ciliates remained alive and in good condition for months, *Ancistruma* proving to be much less sensitive to environmental changes than *Conchophthirius mytili* (Kidder, 1932). The ciliates were obtained for examination by opening the valves of the mussel and washing the contents of the mantle cavity into a Syracuse watch-glass. Under the dissecting microscope individuals were then selected and with a fine pipette placed on coverglasses for examination and fixation.

The fixatives and stains employed were: Schaudinn's, sublimate-acetic, Bouin's, Zenker's and Champy's fluids followed by Heidenhain's and Delafield's hæmatoxylins, the Borrel stain, and the Feulgen nuclear reaction. (The formulæ and methods for both the Borrel stain and the Feulgen reaction may be found in Calkins, 1930.)

The " silver line system " was studied by the silver nitrate impregnation method of Klein (1926*a*, 1926*b*, 1927) and also by a few modifications of it. The most useful modification was to kill the ciliate in osmic acid fumes (10 to 20 seconds). Before the organism became dry a drop of distilled water was added. This was drawn off with a fine pipette, and the process repeated a number of times. Thus the sea salts were removed and the formation of a heavy precipitate, upon addition of the silver nitrate, prevented. A 2 per cent solution of silver nitrate was added and the preparation exposed to the light. The time of impregnation varied with the length of time the organism was exposed to osmic fumes. By watching the process under the dissecting microscope

the reaction was halted when the ciliates had reached the desired darkness. The impregnation usually took from one to three hours. The advantage of killing in osmic vapor is that the shape of the ciliate is retained with very little of the distortion that occurs by simply drying.

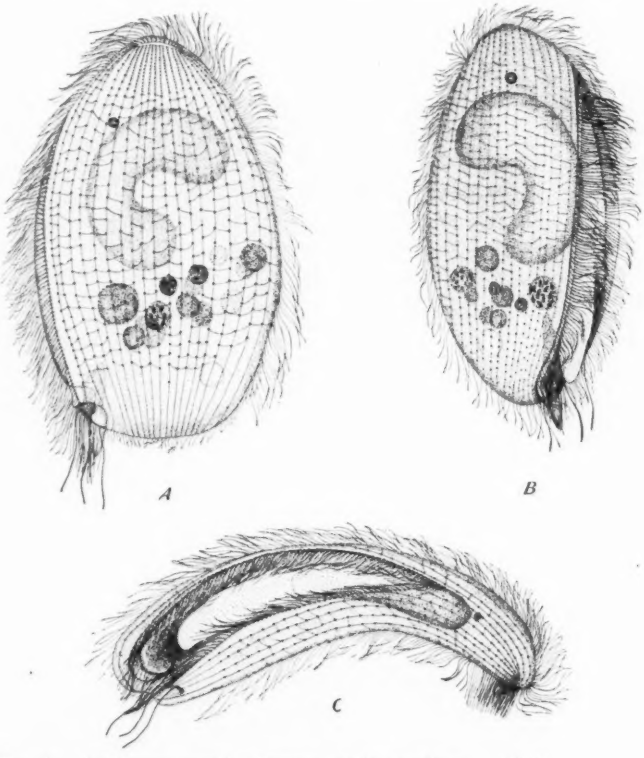

FIG. 1. *Ancistruma mytili.* Camera lucida drawings. × 1000.

A. Ventral view. Schaudinn's: Heidenhain's hæmatoxylin.

B. Dorsal view. Mouth is seen to be slightly dorsal near the posterior end. Zenker's: Mallory triple stain.

C. Lateral view of the right side. At the anterior end may be seen the tuft of straight tactile cilia. Sublimate-acetic: Heidenhain's hæmatoxylin.

For the detailed structure of the ciliary insertions individuals were fixed in Zenker's and Champy's fluids and sectioned. These sections were then stained with Heidenhain's hæmatoxylin or crystal violet-sulph-alizarinate (Benda's alizarin method, Lee's Microtomist's Vade-Mecum, 9th ed., p. 335).

General Features of Ancistruma mytili

The following terms of orientation will be used in dealing with the members of the genus *Ancistruma*. The end directed forward in normal swimming will be called anterior. The concave surface always in contact with the substrate will be called ventral. (For a discussion of this question, see Kidder, 1932.)

The swimming movements of *A. mytili* are very characteristic. In the Syracuse dish the ciliates continuously leave the bottom of the dish and come to the surface film, then immediately return. During these flights they revolve rapidly on a diagonal axis, and have the appearance of moving in a series of jerky somersaults. They exhibit ceaseless activity and seldom remain at rest for more than a short period of time.

Size and Shape

Ancistruma mytili averages 67 μ (52 μ–74 μ) in length and 31 μ (20 μ–38 μ) in width. These measurements were made on fifty living specimens. Fixed material gives measurements of a considerably lower value. The organism is nearly oval in shape when viewed from the dorsal or ventral surfaces (Fig. 1, *A* and *B*). The left margin is slightly more convex than the right. In lateral view the ciliate is seen to be elliptical and bent into an arc, the ventral surface being deeply concave and the dorsal surface convex (Fig. 1, *C*). The extreme dorsoventral flattening has been accurately described and figured by Maupas (1883) and De Morgan (1925).

The peristomal groove starts on the extreme right margin near the anterior end and extends to the posterior end of the organism. It is narrow and pointed anteriorly and widens gradually toward the posterior end. It terminates in the wide mouth situated in a slightly dorsal position. The dorsal edge of the peristome swings down in an even arc about the mouth. The ventral edge, however, curves sharply to the left in an inverted arc, forming a pointed flap just under the mouth. This flap and the slightly dorsal position of the oral aperture make it exceedingly difficult to study from the ventral surface. The floor of the peristomal groove is thrown into a fold which has the appearance of a tongue and reminds one of the shelf in the peristome of *Conchophthirius mytili* (Kidder, 1932). This tongue extends to the oral aperture, becoming more protruding toward the posterior end (Fig. 1, *B*).

The body of the ciliate is covered with a thick elastic pellicle. No contractility is exhibited, but the whole organism may be bent by mechanical forces.

Peripheral Cilia

The peripheral cilia originate from rather large basal bodies arranged in longitudinal rows. These rows begin at the anterior field slightly back of the anterior extremity on the ventral surface. They pass backward and around the posterior end, returning on the dorsal surface and around the anterior end to the anterior field. The rows on the ventral surface are more numerous and much closer together than those on the dorsal. In sectioned material this relationship is clearly brought out (Fig. 2). The basal bodies are connected longitudinally by fine coördinating fibers.

The cilia are long and wavy with the exception of a single tuft of tactile cilia at the anterior end on the ventral surface. In this region the basal bodies are small and very numerous, and from each originates a single stiff cilium. The tuft of cilia may be seen easily from a lateral

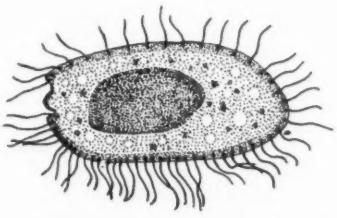

FIG. 2. *Ancistruma mytili.* Camera lucida drawing of a cross-section through the mid region looking from the anterior to the posterior end. Champy's: Crystal-violet. × 1000.

view (Fig. 1, *C*). It may also be seen in life when the organism attaches itself to the coverglass ventral side up. Then each cilium appears as a tiny point against the glass while the other cilia over the body continue to wave. This tuft of cilia is the attaching mechanism and is undoubtedly used in anchoring the organism to the mantle and gills of the host. It often attaches itself by this tuft to the bottom of the Syracuse dish or to pieces of debris.

Peristomal Cilia

Slightly back of the pointed end of the peristomal groove three rows of very long cilia originate. Two rows follow the dorsal edge of the peristome posteriorly and dip down in an arc behind the mouth. The other follows the ventral edge of the peristome and ends just over the point of the ventral flap. The cilia of these three peristomal rows are nearly twice as long as the peripheral cilia and are set exceedingly close

together. When the organism is in motion they are seen to move in waves like undulating membranes. At the posterior ends of these rows arise three still longer and heavier cilia. De Morgan (1925) figures and describes these three cilia. His observations of posterior lobes or dorsal ridges, however, I have been unable to verify in my material although I have looked for them repeatedly. The surface of the organism often does become folded and the lateral edges papillose after remaining on a slide for a short time.

Fibrillar System

The longitudinal coördinating fibers connecting the basal bodies are easily seen after Heidenhain's hæmatoxylin or Mallory's triple stain, but it is with reduced silver that they stand out with great clearness. Successful preparations of the silver line system are relatively easy to obtain using *Ancistruma* as material. The cytoplasm is yellowish and the basal bodies and fibers are black. I have found this method very useful in studying the fine details of the general morphology of this genus in addition to bringing out a system of fine fibers not demonstrated by any other technique.

The fibers are of three types in both *Ancistruma mytili* and *A. isseli:* the longitudinal coördinating fibers, the transverse or commissural fibers, and the net of fine fibers in the peristomal region (Fig. 3, *A*, *B*, and *C*).

The longitudinal fibers of *A. mytili* originate in a clear, bar-like area in the ventral anterior region, the anterior field (Fig. 3, *A* and *C*). This area does not appear to be fibrillary, as it remains unstained after all the methods I have employed. The basal bodies from which the stiff tactile cilia arise are small and very close together. Posterior to this region the basal bodies are larger and farther apart. The basal bodies of the three peristomal rows of cilia are large and numerous.

The transverse fibers are delicate and only occasionally can be seen after hæmatoxylin stain. They stand out quite clearly after silver impregnation, however. They are very irregular in their distribution. I have never seen any in the region of the tactile cilia and only occasional pairs of basal bodies in the posterior region show cross connections. They are most numerous in the mid region of the body on both dorsal and ventral surfaces.

Figure 3, *A*, represents an organism in which the impregnation was exceedingly clear and delicate. In one region on the left side of the organism a few fibers are seen lying between the rows of basal bodies. These may represent the interstrial fibers described by Pickard (1927) in *Boveria teredinidi*. On the other hand, they may represent a deep-lying network of the same type regularly seen in the peristomal region,

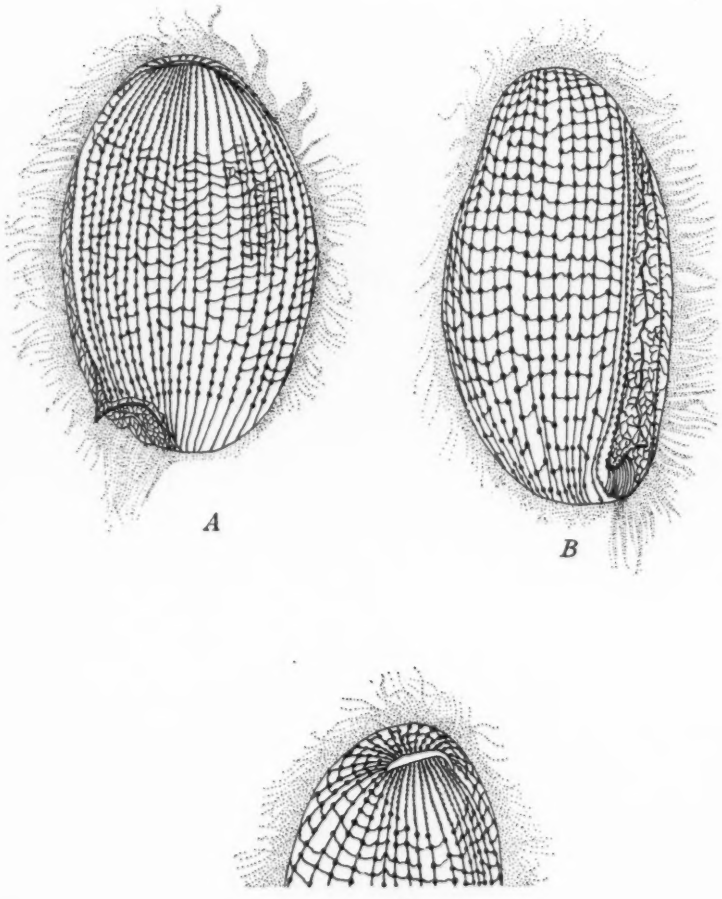

FIG. 3. *Ancistruma mytili.* Camera lucida drawings of organisms impregnated with silver nitrate. × 1000.

A. Ventral view. Note the fine interstrial (?) fibers appearing on the left side of the organism.

B. Dorsal view. The net of fibers in the peristome are readily seen in this view. Note also the row of fine fibers below the mouth.

C. Anterior end of an organism turned slightly on its right side. Note bar-like anterior field.

their presence being masked by the heavy pellicle in the body region so that they appear only under exceedingly favorable conditions.

The whole peristomal region, even into the mouth, is supplied with a net of very fine fibers. These fibers spread in all directions and seem to connect directly with the basal bodies of the peristomal cilia.

A number of fine fibers connecting the inner dorsal row of peristomal cilia to the outer dorsal row may be seen just posterior to the mouth. They seem to be quite distinct from the net system and probably are in the nature of concentrated transverse or commissural fibers (Fig. 3, *B*).

Nuclei

Ancistruma mytili contains one very small micronucleus located well up in the anterior end. It is spherical and, in the interphase, always stains intensely with any of the basic dyes. It is readily observed as the cytoplasm in the anterior region is very clear and free from food particles.

The macronucleus is relatively huge and very characteristic in shape and position. It is clearly visible in the living organism. It resembles a large, curved sausage, having its convex side always toward the peristome. In some organisms the degree of curvature is less than in others. Maupas (1883) and De Morgan (1925) both sketch the macronucleus of *A. mytili* entirely too small in proportion to the size of the organism. Moreover, De Morgan represented the convex surface as directed toward the left side instead of toward the peristome. I have never found this to be the case in my material.

The macronucleus of *A. mytili* in the resting stage is seen to be composed of very compactly placed chromatin granules. These take the Feulgen reaction intensely and stain clearly with any of the basic dyes.

Vacuoles

Many food vacuoles are found in the posterior portion of the body. These are filled with bacteria and small particles taken from the water brought in by the mussel. Many of the vacuoles contain algæ.

There is one contractile vacuole usually situated well to the left side near the posterior end. It is quite regular in its contractions, filling slowly and emptying rapidly. I have never been able to detect any definite and persistent pore in the periplast through which this discharge takes place. As the organism becomes enfeebled on the slide the rate of discharge decreases until finally it ceases and the vacuole reaches enormous proportions. This persists for one to three minutes before cytolysis takes place.

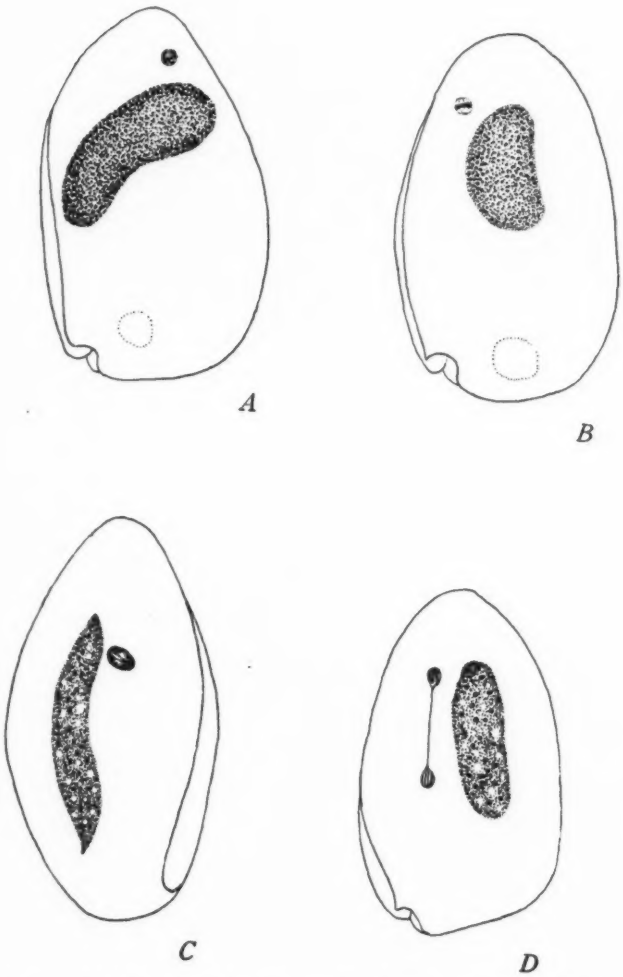

FIG. 4. Division of *Ancistruma mytili*. Camera lucida drawings. × 1000.
A. Micronucleus in prophase. Schaudinn's: Feulgen.
B. Metaphase. Macronucleus straightening. Zenker's: Feulgen.
C. Late anaphase. Macronucleus elongate. Schaudinn's: Heidenhain's hæmatoxylin.
D. Telophase. Schaudinn's: Heidenhain's hæmatoxylin.

Division of Ancistruma mytili

The division of *A. mytili* appears to be regular and of the usual ciliate type. The micronucleus is too small to allow detailed observation of inner structure. I shall outline the process briefly.

The micronucleus initiates fission. Its chromatin becomes less dense and appears as masses in a lightly-staining matrix. The macronucleus straightens out with its long axis corresponding to the long axis of the body (Fig. 4, *A*). There is very little swelling of the micronucleus and the metaphase is formed by an apparent condensation of chromatin into a plate (Fig. 4, *B*). This plate of chromatin divides and the two halves move to opposite poles of the then elongated micronuclear spindle (Fig. 4, *C*). The macronucleus lengthens considerably and becomes filled with deeply-staining granules and clear areas. This reminds one of the condition of the macronuclei of *Uroleptus halseyi* (Calkins, 1930). The micronucleus now divides, pulling out a long connecting strand between the two halves (Fig. 4, *D*). The connecting strand disappears and the two daughter micronuclei migrate to opposite ends of the cell. The macronucleus, meanwhile, begins to constrict and pull into a dumbbell shape (Fig. 5, *A* and *B*). In the clearer area between the dividing halves many large granules are regularly seen (Fig. 5, *B*), but as the separation proceeds these granules of chromatin are drawn up into the daughter halves. The separation of the daughter halves is clean, and the daughter macronuclei round up and become compact. The large granules and vacuoles disappear, and the ciliate undergoes plasmotomy (Fig. 5, *C*). The macronuclei of the daughter ciliates then elongate and assume their characteristic sausage-like shape.

General Features of Ancistruma isseli

A Syracuse dish containing the contents of the mantle cavity of *Modiola modiolus* is a scene of chaos, when viewed through the dissecting microscope. *Ancistruma isseli* exhibits more speed in swimming than does *A. mytili*. The ciliates are exceedingly numerous in most mussels and tend to swim in one direction, in contrast to the jerky flights of *A. mytili*.

Size and Shape

Fifty specimens of *Ancistruma isseli* gave an average length of 77 μ (70 μ–88 μ) while the average width was 42 μ (31 μ–54 μ). These averages are higher than those obtained for *A. mytili* although the ranges overlap.

The shape of *A. isseli* is roughly similar to that of *A. mytili*, but the former is more pointed at both the anterior and posterior ends. The

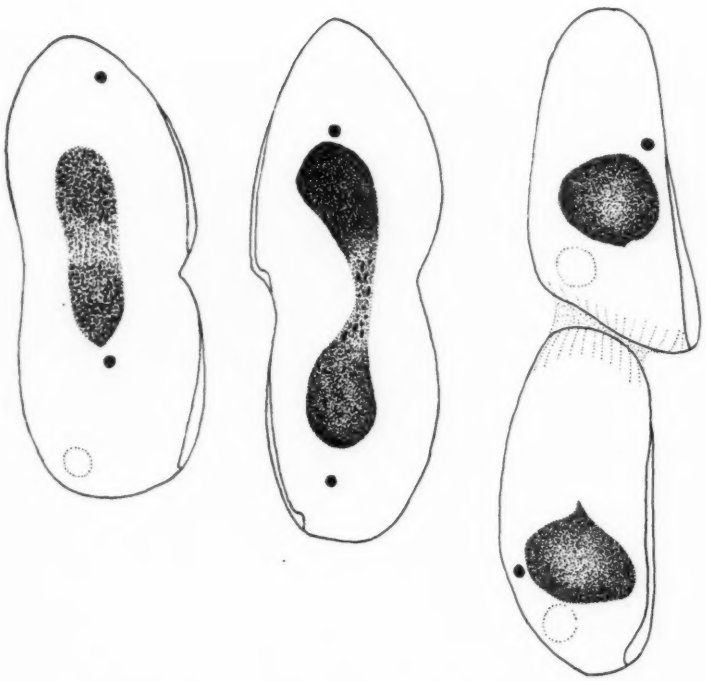

FIG. 5. Division of *Ancistruma mytili*. Camera lucida drawings. × 1000.

A. Daughter micronuclei are again compact and have moved apart. Macronucleus showing division plane. Reorganized peristomes visible. Zenker's: Feulgen.

B. A little later. Macronucleus dumb-bell-shaped. Schaudinn's: Delafield's hæmatoxylin.

C. Division of ciliate nearly completed.

right margin is only slightly convex while the left margin is greatly convex, the widest point being in the posterior third of the body (Figs. 6 and 7).

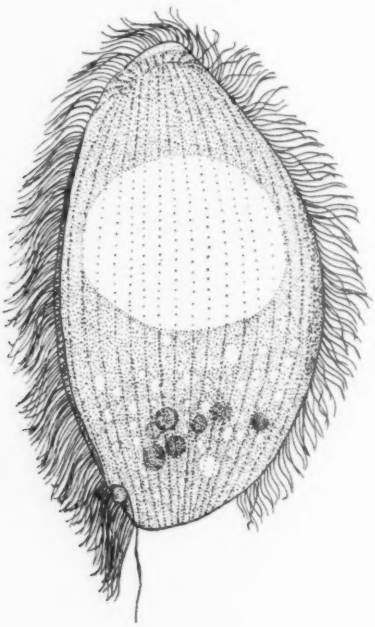

FIG. 6. *Ancistruma isseli.* Ventral view of organism drawn from life at approximately 1000 diameters. The macronucleus is extremely large and clear.

In lateral view *A. isseli* and *A. mytili* resemble one another closely. Both are dorso-ventrally flattened, concave ventrally, convex dorsally, and both possess attaching cilia. *A. isseli,* however, possesses a much enlarged anterior field, at the extreme anterior end. This field is in the form of a clear bubble in the periplast and is seen especially well when the ciliate is flattened under a coverglass. It is usually visible in fixed specimens (Fig. 8, *A* and *B*). The peristomes of the two species are very similar, the pointed flap under the mouth being less prominent, however, in *A. isseli.*

Cilia and Fibrillar System

The peripheral and peristomal cilia of *A. isseli* are long and wavy as in *A. mytili.* The tactile tuft is much less prominent and less extensive

in the former species. The ciliary lines do not pass completely about the posterior end but center at an easily visible suture. This suture is only observed from the dorsal side in organisms that are slightly flattened. After silver impregnation it appears as a black fiber (Fig. 8, *B*).

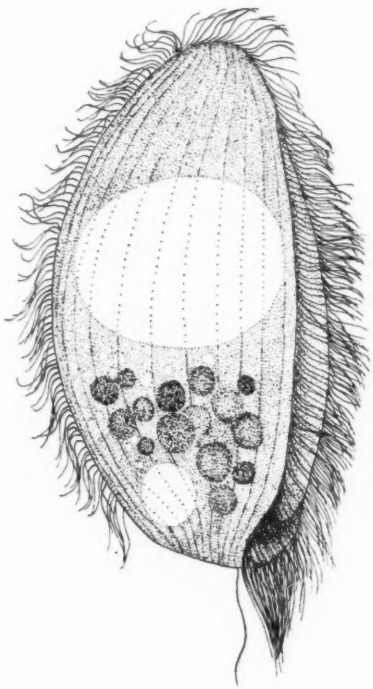

FIG. 7. *Ancistruma isseli.* Dorsal view of living organism at approximately 1000 diameters. Mouth, contractile vacuole and macronucleus visible.

The network of fibers in the peristomal region is much coarser and less extensive than in *A. mytili* and I have never observed any fibers (interstrial?) between the lines of cilia on the body surface. The fine fibrillar connections between the outer and inner dorsal rows of peristomal cilia seem to be lacking in *A. isseli.* Instead the outer dorsal peristomal fiber dips down and forward to join the end of the inner dorsal peristomal fiber just ventral to the mouth.

The fibers of the ciliary system seem to be all interconnected in *A. isseli* through the peristomal net system. The whole fibrillar organiza-

tion is certainly very similar to that described by MacLennan and Connell (1931) for *Eupoterion pernix*.

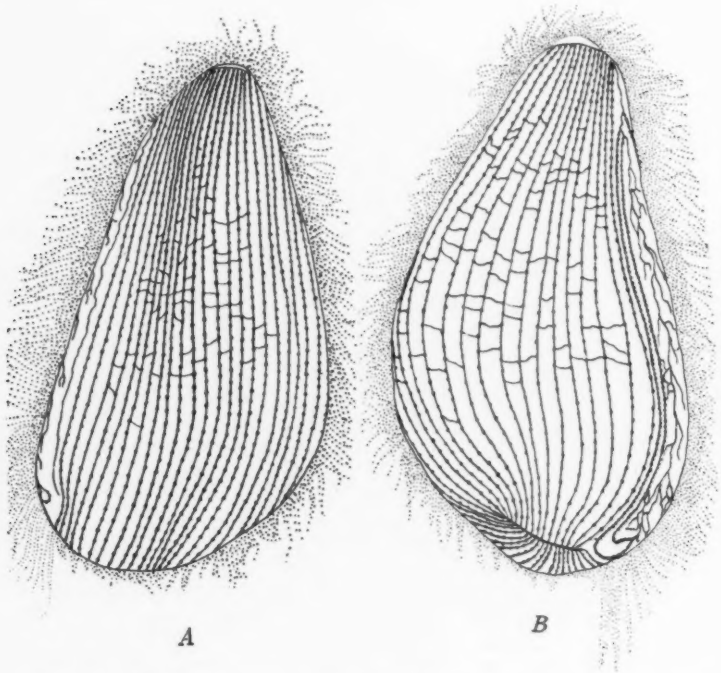

FIG. 8. *Ancistruma isseli*. Silver nitrate impregnations. Camera lucida drawings. × 1000.

A. Ventral view.

B. Dorsal view showing posterior suture. Note also the clear bubble-like anterior field.

Nuclei and Vacuoles

The micronucleus of *A. isseli* is a small sphere situated in the anterior fourth of the body. It is identical with the micronucleus of *A. mytili* as far as can be determined. The macronucleus of *A. isseli* is very distinctive, however. It is clearly visible in life as an enormous sphere occupying the center of the body (Figs. 6 and 7). Some shrinkage in the size occurs upon fixation. The macronucleus is of the massive type but it possesses many spherical deeply-staining chromatin granules among the smaller packed granules (Fig. 9, *A*).

Issel (1903) reports the macronucleus to be fragmented at times into two to seven parts. I have evidence that the spheres so often found in both *Ancistruma isseli* and *A. mytili* are not the result of fragmentation but the reorganization of an ex-conjugant. I shall present this evidence with a discussion of the question in the second of this series of papers.

The food vacuoles are numerous and situated in the posterior third of the body. They are usually filled with the yellow, granular pigment of *Modiola*.

The contractile vacuole is identical in size and position with the contractile vacuole of *A. mytili*.

Division of Ancistruma isseli

The micronucleus of *A. isseli* in division resembles that of *A. mytili*. It is too small to observe with certainty the details of any internal structures. The general sequence of its activity may be seen in Figs. 9, *A*, *B*, *C*, *D*, *E*, *F*, and 10, *A*, *B*, and *C*.

The interesting feature of division is to be found in the macronucleus. As the micronucleus is dividing the macronucleus pulls out into an elongate mass. As this mass begins its constriction two light areas may be seen appearing on either side of the center. These areas are to become the planes of fission of the macronucleus, the heavily-staining mass between them becoming extrusion chromatin (Fig. 9, *D* and *E*). As the two constrictions increase the extrusion mass becomes spherical (Fig. 9, *F*). The two daughter macronuclei now draw apart and round up, pulling out the macronuclear membrane into a long tube flared at the center to accommodate the residual chromatin (Fig. 10, *A*). Very soon the connections between the daughter nuclei are severed and the residual mass contracts (Fig. 10, *B*). The daughter macronuclei round up and plasmotomy proceeds. The residual mass, meanwhile, becomes very loosely granular and loses its staining capacity. By the time the daughter organisms are ready to separate this mass, now in the cytoplasm of one of the daughter organisms, has nearly disappeared (Fig. 10, *C*).

This type of macronuclear action almost exactly parallels that found in *Conchophthirius mytili* (Kidder, 1932) and is quite different from that of *Ancistruma mytili*, described above. The relative amount of chromatin cast out at each division is more than in *Conchophthirius*, but the method of disintegration and absorption into the cytoplasm is the same.

Discussion

Issel's (1903) description of a commensal of *Modiola barbata* under the name of *Ancistrum mytili* was not wholly satisfactory to him. He

states that he had some doubts about its identity with the *Ancistrum mytili* of Maupas, mainly because of the spherical macronucleus. But after examining some ciliates from a number of *Mytilus edulis* (obtained from a merchant) he found all gradations from the sphere to the bent type described by Maupas. He attributed this bent appearance to the beginning of fragmentation.

Kahl (1931) recognizes the distinct difference between the two types and names the ciliate from *Modiola barbata*, *Ancistruma isseli*. From the present study I think it possible to establish the correctness of Kahl's statements, and the validity of his species, as I believe the ciliate here described from *Modiola modiolus* to be identical with the ciliate from *Modiola barbata*.

The main points of difference between *Ancistruma mytili* and *A. isseli* are as follows: (1) Size: *A. isseli*—average 77 $\mu \times 42\ \mu$; *A. mytili*—average 67 $\mu \times 31\ \mu$. (2) Macronucleus: *A. isseli*—spherical; *A. mytili*—sausage-shaped. (3) *A. isseli* has posterior suture; *A. mytili* has no posterior suture. (4) Division: *A. isseli* casts out a mass of macronuclear chromatin at division; *A. mytili* divides sharply with no chromatin extrusion.

The genus *Ancistruma* shows many affinities to the Conchophthiridæ. The peristome of *Ancistruma* is like *Conchophthirius mytili* in that it possesses a shelf or raised portion which protrudes between the rows of peristomal cilia. The motor organization is similar to that of *Eupoterion* as described by MacLennan and Connell (1931). In fact, the main distinctive difference between the members of the Conchophthiridæ (*Conchophthirius*, *Cryptochilum*, and *Eupoterion*) and the genus *Ancistruma* is the possession of the tactile tuft of cilia by the latter.

The "silver line system" first described by Klein (1926) was thought by him to represent a system of nervous connections and coordinating controls. It is evident from the present study that fibers generally thought to be coördinating or conductive in function (longi-

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FIG. 9. Division of *Ancistruma isseli*. Camera lucida drawings. $\times 1000$.

A. Interphase showing compact micronucleus and large spherical macronucleus. Sublimate-acetic: Heidenhain's hæmatoxylin.

B. Micronucleus in metaphase of mitosis. Sublimate-acetic: Heidenhain's hæmatoxylin.

C. Telophase. Sublimate-acetic: Feulgen.

D. Daughter micronuclei drawing apart. Macronucleus elongating. Sublimate-acetic: Heidenhain's hæmatoxylin.

E. Later stage. Note two constrictions in the macronucleus. These represent the division planes while central mass is the residual chromatin. Gilson-Carnoy's: Borrel.

F. Later stage in division of macronucleus. Gilson-Carnoy's: Feulgen.

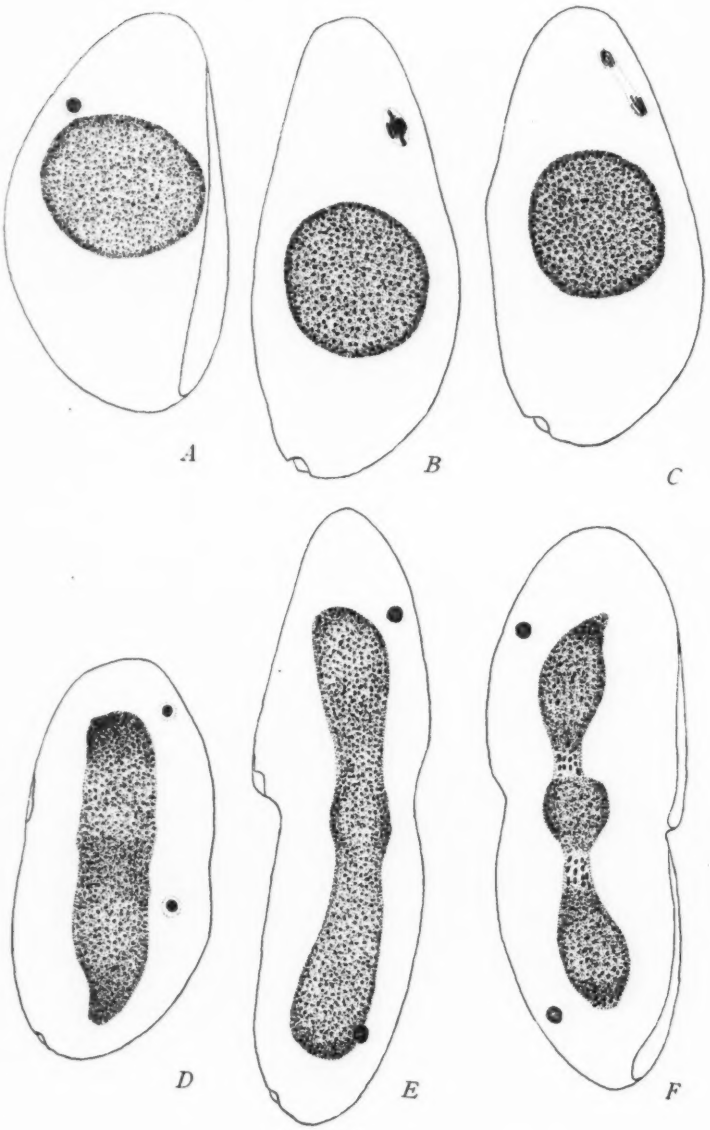

FIGURE 9

tudinal fibers) are stained by the reduced silver after this method. It seems logical to suppose that the network of fine fibers in the peristomal region of *Ancistruma* is conductive and serves to connect this sensitive area with the organelles of locomotion.

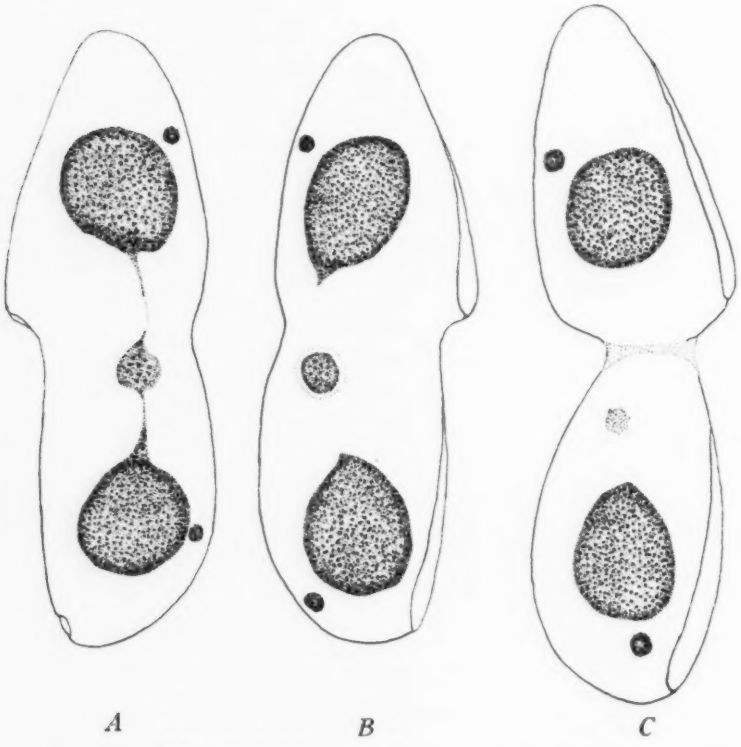

FIG. 10. Division of *Ancistruma isseli*. Camera lucida drawings. × 1000.

A. Late division of macronucleus. Extrusion chromatin still within macronuclear membrane. Gilson-Carnoy's: Borrel.

B. Later. Daughter macronuclei separate. Residual mass of chromatin free in cytoplasm. Sublimate-acetic: Feulgen.

C. Daughter macronuclei rounding up. The residual chromatin is being absorbed into the cytoplasm of posterior daughter organism. Sublimate-acetic: Feulgen.

It seems strange that two such similar forms as *Ancistruma mytili* and *A. isseli* should show such a decided difference in macronuclear division. If this chromatin elimination is a purification process as sug-

gested by Calkins (1930), then there must be some fundamental difference between the two species in the chromatin reorganization during division. *Ancistruma mytili* must be able to eliminate its nuclear waste products gradually or at least in a manner as yet impossible of visual detection. Whether or not this process of chromatin elimination has any phylogenetic significance is unknown. Perhaps some light may be thrown on this question when sufficient data have been collected and analyzed. The Conchophthiridæ and the Ancistridæ seem to be the most favorable material for such a study.

Summary

1. *Ancistruma mytili* Quenn., a holotrichous ciliate commensal in the salt water mussel *Mytilus edulis,* and *Ancistruma isseli* Kahl, a holotrichous ciliate commensal in the solitary mussel *Modiola modiolus* are described.

2. Observations are made from both living and fixed material.

3. The motor system of each consists of long peripheral cilia, a tuft of tactile cilia, and three rows of peristomal cilia all supplied with interconnected basal bodies. Net-like systems of coördinating fibers occur in the peristomal regions.

4. Each species possesses one micronucleus, which is small and compact.

5. The macronucleus of *A. mytili* is sausage-shaped while that of *A. isseli* is spherical. Both are very large.

6. During division the micronuclei divide in the usual manner. They are too small to allow observation of detail.

7. The macronucleus of *A. mytili* divides evenly and cleanly, while that of *A. isseli* eliminates a large ball of chromatin at each division.

8. The differences between the two species are discussed.

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GRUBERIA CALKINSI SP. NOV., A BRACKISH-WATER CILIATE (PROTOZOA, HETEROTRICHIDA) FROM WOODS HOLE, MASSACHUSETTS

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While working at the Marine Biological Laboratory, Woods Hole, Mass., last summer, I had the opportunity to collect many interesting forms of Protozoa from a small brackish-water deposit situated near Gardiner Road, locally known as "Lillie's Ditch."

Among those forms studied I found a large ciliate belonging to the family Plagiotomidæ, exceptionally abundant during August and September. At that time I was unable to place it in any of the genera previously described.

As may be seen (Fig. 1) this animal has a superficial similarity to *Spirostomum*. It differs, however, in some important respects, particularly in the possession of many vacuoles (possibly contractile) which are scattered throughout the body, instead of the characteristic large posterior contractile vacuole of *Spirostomum*, as seen in the typical species.

Gruber (1884) described a new species of *Spirostomum* (*Sp. lanceolatum*) while studying the Protozoa from the port of Genoa, Italy. Gruber's form presents striking similarity to the animals which I collected from Lillie's Ditch, but differs mostly in respect to the morphology of the nucleus. It also lacks the characteristic posterior contractile vacuole of *Spirostomum*.

Some years ago De Morgan (1923–1925) found in the sea-water tanks at Plymouth Laboratory some specimens of *Spirostomum lanceolatum*. His description, far more accurate and complete than that offered by Gruber (1884), shows some points of difference from the latter and a closer similarity in nuclear structure to the animals found at Woods Hole. However, the differences are sufficient to separate perfectly the two species.

Florentin (1899) described under the name of *Spirostomum salinarum* a brackish-water form, which he found in Lorraine, France, as having a yellow color, non-contractile body, and living in media of high saline concentration. The presence of small, independent, granular nuclei, and a typical posterior contractile vacuole, are characters which

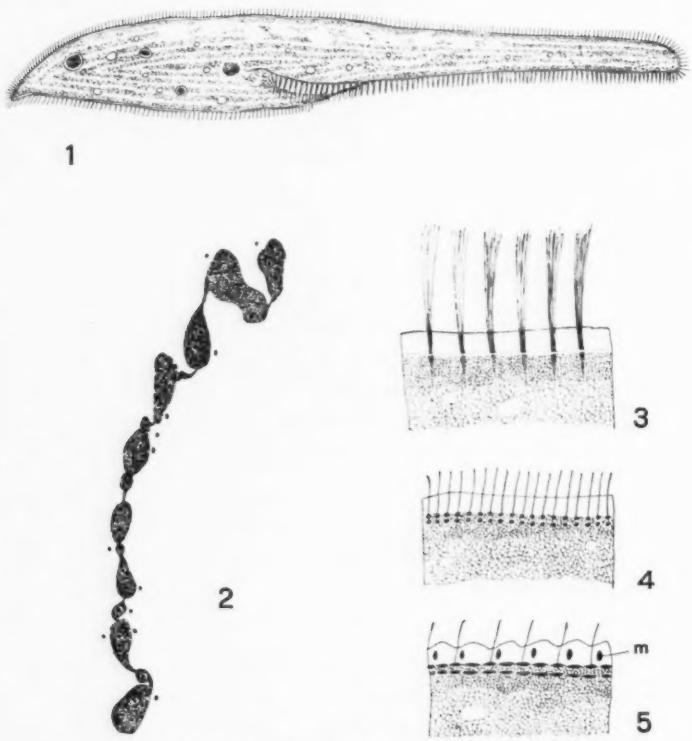

FIG. 1. *Gruberia Calkinsi* sp. nov. From a living specimen.

FIG. 2. Nuclear apparatus. Hg_2Cl, Heidenhain's hematoxylin.
Zeiss Comp. 15 ×
Zeiss Apo. 90 ×, 1.3 n. a.

FIG. 3. Longitudinal section (5 microns thick) showing membranelles. Hg_2Cl, Heidenhain's hematoxylin.
Zeiss Comp. 15 ×
Zeiss Apo. 90 ×, 1.3 n. a.

FIG. 4. Longitudinal section (5 microns thick) of the periplast, showing ciliary arrangement. Hg_2Cl, Heidenhain's hematoxylin.
Zeiss Comp. 15 ×
Zeiss Apo. 90 ×, 1.3 n. a.

FIG. 5. Transverse section (5 microns thick) of the periplast; *m*, myonemes. Hg_2Cl, Heidenhain's hematoxylin.
Zeiss Comp. 15 ×
Zeiss Apo. 90 ×, 1.3 n. a.

are quite sufficient to differentiate this species from the Woods Hole animals.

After careful consideration, I reached the conclusion that I had obtained a new genus of the family Plagiotomidæ.

Three weeks ago, Professor Gary N. Calkins was kind enough to call to my attention the last fascicle (1932) of Kahl's monumental work on the Infusoria. This book has just come out of press, and presents a consideration of the Heterotrichida. This author separates the animal described by Gruber (1884) from the genus *Spirostomum* and creates the new genus *Gruberia* for it, and another species which he calls *Gr. uninucleata*. He also transfers Florentin's *Spirostomum salinarum* to the genus *Blepharisma*. I fully subscribe to these changes, which I think are justified.

The animal which I have studied differs in some respects from those described by Kahl in his genus *Gruberia*, and, evidently, is not either of the species he mentioned. However, the differences are not sufficiently strong to create a new genus, and I prefer to describe the animal found at Woods Hole as a species of the genus *Gruberia*. It has been a great pleasure for me to name it *Gruberia Calkinsi*, as a manifestation of my indebtedness to Dr. G. N. Calkins, not only for his invaluable teachings but also for the kindly interest he has taken in my work, and the many attentions he has shown me during my stay at Woods Hole and Columbia University.

Description

The size varies from 200 to 800 microns in length. The width ranges from one-fifth to one-seventh of the total length.

The animal (Fig. 1) is long and straight, with an obtuse anterior end that is slightly curved toward the ventral side. The posterior end is more or less acutely pointed, and is frequently curved in the same direction as the anterior end.

This general shape is maintained within certain limits. The body is slightly contractile, but the general shape is not altered when contraction occurs. However, some animals may be obliquely twisted on their longitudinal axes.

The peristomial groove is quite long and extends posteriorly in a straight line to two-thirds of the total body length. It is a relatively deep and broad furrow that gradually sinks into the body. On one margin of the peristome there is a line of membranelles, and on the opposite margin is a row of cilia, which are longer than those which may be found in other parts of the body. Sometimes the posterior portion of the peristome may be obliquely twisted to one side.

The mouth, which is fairly wide, leads into a short and narrow pharynx. The membranelles of the peristome continue into the mouth and form a complete spiral within the gullet. This condition is quite similar to that which has been described by Fauré-Fremiet (1912) in *Condylostoma patens.*

The cilia are small and fine, and are arranged in longitudinal rows which extend over the entire length of the body, in lines which follow the general shape of the animal. There are many small vacuoles (possibly contractile) irregularly distributed throughout the body, but they are generally more abundant in the posterior part of the animal.

The macronucleus is of the beaded type, and is situated on the dorsal side of the body. It possesses generally about ten beads.

There are many micronuclei. These are small and irregularly distributed near the beads of the macronucleus, which they generally exceed in number.

I collected all my animals from Lillie's Ditch, in which place occasionally they were exceedingly abundant.

Professor D. H. Wenrich, of the University of Pennsylvania, has shown me some specimens which are apparently the same animal that I have studied. These were collected by him in the Eel Pond, Woods Hole, Mass.

Structure

My intention is not to make a detailed cytological study of these animals. However, the careful observation of a great number of living and fixed specimens permits me to describe some of their structural details.

Nuclear Apparatus

The macronucleus (Fig. 2) quite markedly resembles that of *Spirostomum ambiguum.* It extends lengthwise through the whole body, excepting the anterior and posterior ends. It is situated dorsally, and immediately below the cortical layer.

In the living specimens the nucleus is easily seen as a series of clear spaces. After treatment with dilute aqueous solution of neutral red, the macronucleus appeared to be enclosed within a very fine membrane. This membrane stains less intensely than do the beads. Each bead is formed of a densely and finely granulated substance, in which lie some larger and more refringent granules.

Slides which were fixed with a saturated solution of mercuric bichloride in 95 per cent alcohol, and stained with Heidenhain's iron hematoxylin, or Kornhauser's modification, show the beads to be com-

posed of a finely granular groundwork in which are dispersed larger, deeply-staining granules, which correspond to the refringent granules mentioned above.

The connecting membrane is clear and definite, but generally does not stain very darkly. This membrane may be likened to a sack which bulges in places wherein the macronuclear beads are contained. The connecting portion between two beads may be narrow and clear, or may contain a deeply-staining spherical granule, which is generally larger than those which are found in the beads.

With the Feulgen nuclear reaction, large vacuoles appear in the granular background of the beads. These vacuoles occupy the same position in the bead as the large refringent granules that were demonstrated by staining with neutral red and the large, deeply-staining granules seen in specimens that were stained with iron hematoxylin.

I have not been able to find a single dividing animal, although I have observed more than one hundred individuals. For this reason I cannot treat of the behavior of these granules during nuclear division.

However, the fact that these granules, which stain with iron hematoxylin, do not appear in specimens that have undergone the Feulgen reaction, and the fact that their number and size vary greatly, have led me to believe that they may be homologous with what Calkins (1930) calls " X-granules." The small Feulgen and hematoxylin-staining granules in the macronuclear beads may be homologous with the " C-granules " described in *Uroleptus Halseyi* by the same author.

The micronuclei are generally no more than one or one and a half microns in diameter (a single bead of the macronucleus measures from 10 to 40 microns on its major axis). They are scattered near the macronucleus beads, but may sometimes be imbedded in a surface cavity of the latter.

With Heidenhain's hematoxylin they stain very well, but are never so dark as the supposed X-granules of the macronucleus. The Feulgen reaction demonstrates them as poorly-staining bodies. This probably is an indication of low nucleic acid content.

Membranelles

In the living specimens these structures are narrow, triangular in shape, and situated very close to each other; each measures about 30 microns in length.

In stained specimens, a membranelle is always broken up into its constituent cilia (Fig. 3). It passes through the periplast in the form of a slender, relatively deeply-staining shaft, which emerges into the thin ectoplasmic layer, and there assumes the form of a slender cone, which

has its base nearest the periplast. There appears to be a small space which separates the shafts from the cones of the membranelles, and the entire ectoplasmic layer from the periplast. This space may be a result of fixation.

The membranelles are strikingly similar in structure to those described by Maier (1903) in *Stylonychia*.

Cilia

The cilia are small and thin, and are arranged in rows which extend all along the animal's body. In longitudinal sections (Fig. 4) they are uniformly distributed throughout the periplast, and arise from spherical basal granules, which are one micron in diameter. These small bodies form a row immediately below the periplast, which is 2 microns thick. A second row of similar granules appears directly beneath them. As far as I could determine, there was no connection between these two rows.

In transverse sections (Fig. 5), the cilia are more separated than in longitudinal sections (a space of three or four microns exists between two consecutive cilia). They are inserted in the lower parts of the undulating periplast, and have the same relations to the double row of granules as shown above. The granules appear to be fusiform in shape, with their longest axes parallel to the periplast. They are situated very closely to each other, and may be connected by pointed ends.

The same apparent lack of connection between the two rows, as shown in longitudinal sections, appears in transverse sections.

Myonemes

Slender, refringent fibers which extend lengthwise over the body can easily be seen in the animal. These are about one micron in diameter and extend parallel and very close to the ciliary rows. They are distinct at the middle body region, and sometimes anastomose at the ends.

I interpret these fibers as myonemes, although the animal is only slightly contractile. And I suppose that the interciliary bodies, which in transverse sections appear within the hyaline periplast (Fig. 5, *m*), are cross-sections of these fibers.

These fibers are similar in position to the myonemes described by Bovard (1907) in *Condylostoma patens*.

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THE OCCURRENCE OF THE HUMORAL CHROMATOPHORE ACTIVATOR AMONG MARINE CRUSTACEANS

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The chromatophore-activating principle of the crustacean eye-stalk has been found in *Palæmonetes* by Perkins (1928), in *Crangon* and several species of *Leander* by Koller (1928), in *Macrobrachium* by Smith (1930), in *Praunus* by Koller and Meyer (1930), and in *Callinectes* by Perkins and Kropp (1932). Already indicated as present in several families of decapods, the hormone was looked for in as great a variety of crustaceans as could be obtained in the vicinity of the Mt. Desert Island Biological Laboratory, at Salisbury Cove, Maine. We had available seven different species of marine crustaceans belonging to as many different families. Six were decapods—*Crago* (= *Crangon*) *borcas, Pandalus montagui, Homarus americanus, Pagurus longicarpus, Libinia emarginata,* and *Cancer irroratus;* one was a schizopod —*Mysis stenolepis.*

The eyes were extracted in sea water as has been described previously. Unfortunately, the extracts could not in all cases be tested on the species from which they were derived. This was impracticable with *Homarus, Libinia* and *Cancer* because of the lack of external chromatophores that undergo readily observable changes. *Pandalus* and *Pagurus* did not remain alive long enough under laboratory conditions to permit adequate tests, and *Mysis* was too small for injection in addition to being very fragile and non-resistant. Therefore, *Crago borcas* was used as a test animal for all extracts. Measured amounts of the extract in question were injected into two sets of *Crago* which had previously been adapted to white and black backgrounds respectively. In all cases the chromatophores of *Crago* were examined microscopically before and after the eye extract was injected in order to determine the degree of contraction or expansion. Control animals, similarly adapted, received like amounts of sea water, and still others received the same amount of the donor's abdominal muscle similarly extracted in sea water. Following injections, the animals were returned to the background on which they had become adapted; *i.e.*, white-

adapted *Crago,* injected with eye extract, were returned to the white background, and black-adapted *Crago* to the black background. We also took the opportunity to repeat the experiments of Koller (1928) in which he reports the presence of a chromatophore-expanding material from the rostral region of *Crangon.*

Every extract of eye-stalks tested induced a condition in black-adapted *Crago* indistinguishable from the white-adapted state. The effect on the white-adapted animals was to increase the degree of contraction already present. Control animals, whether treated with sea water or extracts of abdominal muscle, showed no comparable effects and in most cases were wholly unaffected. Regarding the action of the rostral region extract we must report completely negative results, as has been reported by Perkins and Snook (1931). In no instance could we obtain a chromatophore-expanding action following the injection of this extract into white-adapted *Crago* although care was taken to prepare the material from the region designated and in the manner prescribed by Koller.

The results are given in Table I. No standard has yet been devised for the assay of the crustacean eye hormone. Pending such an assay we adopted a procedure in the preparation and use of the extracts which was designed to give extracts that would be broadly comparable in potency. This was not done, however, with the intention of obtaining at this time quantitatively comparable extracts of known strength, since the potency of a given extract is undoubtedly related to the total mass of the eye and stalk which we were not able to consider. The extract was prepared from a known number of eye-stalks and a measured amount of medium in such a way that 1 cc. of the final extract possessed the hormonal potency of 40 eye-stalks. The quantity injected was 0.1 cc., representing 4 eye-stalks. This was found to be well above the minimum dosage necessary to induce chromatophore response in *Crago* regardless of the species from which the eyes came. Indeed Koller (1930) estimates that the hormone is still active in a 1 : 100,000 solution. With the dosage used, the onset of contraction occurred in one minute with *Pagurus* extract and in five minutes with *Homarus* extract. Maximum contraction was reached three to four minutes after injection with *Pagurus* extract, and within ten minutes of injection with *Homarus* extract. The initial black-adapted state reappeared after ten minutes with *Pagurus* extract and after thirty minutes with *Homarus* extract. All other extracts brought on responses within these limits, except *Mysis,* which called forth a response

TABLE I

Donors	Crago boreas	Pandalus montagui	Cancer irroratus	Homarus americanus	Libinia emarginata	Pagurus longicarpus	Mysis stenolepis
Eye-stalk extract							
Black-adapted *Crago*	contraction	contraction	contraction	contraction	contraction	contraction	contraction
White-adapted *Crago*	no effect	no effect	sharp maximal contraction	no effect	no effect	maximal contraction	no effect
Abdominal muscle extract							
Black-adapted *Crago*	no effect	no effect	no effect	no effect	no effect	no effect	no effect
White-adapted *Crago*	no effect	no effect	no effect	no effect	no effect	slight local expansion	no effect
Rostral region extract							
Black-adapted *Crago*	no effect	no effect	no effect	no effect	no effect	slight local contraction	not tested
White-adapted *Crago*	no effect	no effect	no effect	no effect	no effect	slight local contraction	not tested
Sea water							
Black-adapted *Crago*	no effect	no effect	Slight local contraction	no effect	no effect	slight local contraction	no effect
White-adapted *Crago*	no effect	no effect	slight general expansion	no effect	no effect	slight local expansion	no effect

five to six minutes after the injection, maximum contraction occurred after fifteen minutes, and the original black-adapted condition did not supervene until thirty minutes after the injection. The apparently weaker extract from the eye-stalks of *Mysis* may be associated with the presence of a specifically less potent hormone as well as with the greatly inferior mass of the eye-stalk compared with that of the decapods examined.

Table II represents the occurrence of the crustacean eye-hormone as determined to date.

TABLE II

Family	Species	Author
(Decapoda)		
Crangonidæ	Crangon vulgaris	Koller (1928)
	Crago (=Crangon) boreas	
Palæmonidæ	Palæmonetes vulgaris	Perkins (1928)
	Leander adspersus	Koller (1928)
	Leander serratus	Koller (1928)
	Leander squilla	Koller (1928)
	Macrobrachium acanthurus	Smith (1930)
Pandalidæ	Pandalus montagui	This paper
Nephropsidæ	Homarus americanus	This paper
Paguridæ	Pagurus longicarpus	This paper
Maiidæ	Libinia emarginata	This paper
Cancridæ	Cancer irroratus	This paper
Portunidæ	Callinectes sapidus	Perkins and Kropp (1932)
(Schizopoda)		
Mysidæ	Mysis stenolepis	This paper
	Praunus inermis	Koller and Meyer (1930)
	* Praunus flexuosus	Koller and Meyer (1930)

* Koller and Meyer (1930) report the eye-stalk extract from white-adapted *Praunus flexuosus* as causing slight darkening in white-adapted *Crangon vulgaris*. This reaction is contrary to that induced by eye-stalk extracts of all other crustaceans thus far investigated, and may indicate a greater diversity of action of the hormone within the group than has thus far been anticipated.

The above would indicate that the chromatophore-activating substance of the crustacean eye-stalk is very widely distributed among the members of this group and by no means confined to the decapods. Its occurrence, therefore, cannot be regarded as fortuitous in a single species, but as genetically present probably throughout the group and correlated with the chromatophore effectors in the larval or adult stages.

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THE RELATIVE NUMBERS OF IMMATURE ERYTHROCYTES IN THE CIRCULATING BLOOD OF SEVERAL SPECIES OF MARINE FISHES

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During the course of a study of the reactions of the erythrocytes of fishes to vital dyes (Dawson, 1932) in which brilliant cresyl blue was used to produce patterns of reticulation, it became increasingly obvious that the numbers of immature erythrocytes present in the circulation varied widely in different species. In some fishes the numbers of immature cells were negligible while in others they attained considerable proportions.

These observations were made in the summer of 1931 at the Marine Biological Laboratory on seventeen different species. At that time blood was routinely drawn from the heart. Later it seemed possible that the source of the blood samples might in some measure be responsible for the variations encountered, since in many of the lower vertebrates the final differentiation of a considerable number of erythrocytes commonly takes place in the intertrabecular spaces of the ventricle. Variations in the depth of penetration of the needle might then be suspected as a possible cause of the variations in the number of immature cells, depending on whether the tip of the needle stopped in the intertrabecular spaces or passed into the main channel of the ventricle.

Consequently it was decided to repeat the study, drawing samples of blood from both the caudal vessels and the heart. In the summer of 1932 it was possible to re-study the blood of many forms which had been available in 1931 as well as to extend the study to include several additional species. In no case, however, was the blood from the heart and the caudal vessels found to vary appreciably in the number of immature erythrocytes present. Accordingly, it was concluded that the earlier observations were significant and that the blood of the different species of fishes may show specific differences in the proportions of immature cells present in the circulation.

Information on this subject appears to merit some attention. The erythrocytes of fishes are widely used by physiologists in studies on respiration and hemolysis, and the varying number of immature cells present should be taken into consideration when comparisons of the behavior of the blood of different fishes are made.

Material and Methods

As previously stated, these studies were carried on at Woods Hole in the summers of 1931 and 1932. The blood of the fishes studied in 1931 was drawn only from the heart, while in 1932 samples were taken from both the heart and caudal blood vessels. In many instances it was possible to secure in 1932 specimens of species studied the previous year, but in a few cases some species were not obtainable in the second summer. On the other hand, several additional species were available in 1932. In all, the proportion of immature cells in the circulating blood of twenty species was determined. Freshly-drawn blood was prepared by three different methods. Supravital preparations were made by adding a small drop of blood to a slide previously filmed with a saturated solution of brilliant cresyl blue in absolute alcohol. A circular coverslip was added and the preparation sealed with warm vaseline. In order to secure by this method slides on which accurate counts can be made, it is necessary to add the proper amount of blood, just enough so that when the coverslip is added the blood film will be one cell thick and barely reach the margin of the cover. Preparations which do not fulfill these requirements are unsatisfactory for quantitative studies.

More permanent preparations, also demonstrating the patterns of reticulation, were made by mixing the blood thoroughly with an oxalated solution of brilliant cresyl blue in cold-blooded Ringer's solution (Cook, Meyer and Tureen, 1931). A one per cent solution of brilliant cresyl blue and one per cent solution of potassium oxalate were used in the proportions of one drop of oxalate to five drops of stain. Equal parts of blood and oxalated stain were thoroughly mixed in a capillary pipette, spread thinly on a slide, and stained lightly by Wright's technique. In addition, ordinary smears stained with Wright's were employed to demonstrate the varying degrees of basophilia and polychromasia of the immature cells.

In general it was found that more accurate counts could be made when the patterns of reticulation were adequately developed with brilliant cresyl blue. Corresponding counts of immature cells in blood stained only by Wright's method ran uniformly lower, probably due to the fact that fatigue occurs much more rapidly when the observer has to evaluate continuously the varying degrees of basophilia and polychromasia present. Reticulation patterns, on the other hand, are definite and the amount of reticulation present is much more readily determined.

As shown in an earlier study (Dawson, 1932), remnants of the reticulation patterns persist in practically all the mature erythrocytes of fishes. The degree of persistence varies somewhat in different species

and also in the individual erythrocytes of the same species. When erythrocytes of varying degrees of differentiation are present, the distinction between maturity and immaturity must at times be a relatively arbitrary one. In this regard the slides doubly stained with brilliant cresyl blue and Wright's stain proved valuable, since the amount of reticulation and the degree of basophilia, polychromasia, or eosinophilia present in the individual cells could promptly be correlated.

For the purposes of this study the various types of erythrocytes encountered were arbitrarily divided into five classes as follows: (1) Mature erythrocytes, with the reticular material in scattered granules or isolated, irregular filaments and stained orthochromatically with eosin in Wright's; (2) less mature cells with the reticular material forming a loose, partially fragmented reticulum, staining orthochromatically with eosin or exhibiting a slight polychromasia; (3) younger cells with an open-meshed but practically complete reticulum and definitely polychromatic; (4) still younger cells with a close-meshed, complete reticulum exhibiting a decided basophilic tint when differentially stained; (5) young cells with the reticular substance massed in a dense, almost granular pattern, presenting little evidence of the true reticulation pattern. These cells give a definite basophilic reaction but are rarely seen except in the elasmobranchs.

Counts were made under oil immersion at a magnification of 900 diameters. In order to facilitate counting, a small coverslip was hand-ruled in India ink with a fine pen into areas 2.5 mm. square. This was used as an ocular micrometer at the level of the diaphragm. Four such areas could be used in the field of the microscope and their size was such that the number of erythrocytes seen in an individual square was small enough to be counted readily without any uncertainty. In 1931 only one sample was drawn from the heart but in 1932 an additional sample was drawn from the tail of each fish and two supravital preparations of each sample were counted. Twenty fields of four squares each were taken and the results averaged. In practice this meant that about one thousand cells were counted from each sample. These values were also checked by similar counts made on preparations doubly stained with brilliant cresyl blue and Wright's stain and on preparations stained with Wright's alone. The number of fishes of each species examined was not constant and data on this point will be included in the description of the findings.

Results

On the basis of the number of immature erythrocytes present in the circulation, the twenty species of fish fall naturally into four groups.

TABLE I

Counts of the several classes of erythrocytes present in the circulation of twenty species of fishes studied at Woods Hole during 1931 and 1932. Only the averages are given. Counts are recorded as "few" when no more than ten were encountered in two supravital preparations and "rare" when less than five were seen.

Groups	Species	Number of Individuals 1931	Number of Individuals 1932	I Isolated Granules and Filaments	II Fragmented Reticulum	III Open-meshed Reticulum	IV Close-meshed Reticulum	V Dense Granular Reticulum
				per cent	*per cent*	*per cent*	*per cent*	*per cent*
I	Mackerel, *Scomber scombrus* Linnaeus	4	2	83.8	3.3	4.3	8.6	0.0
	Menhaden, *Brevoortia tyrannus* (Latrobe)	4	2	83.5	3.0	2.4	11.1	0.0
	Alewife, *Pomolobus pseudoharengus* (Wilson)	4	0	83.3	3.6	2.2	10.9	0.0
	Summer Herring, *Pomolobus æstivalis* (Mitchill)	0	2	79.2	6.5	4.1	10.2	0.0
	Bluefish, *Pomatomus saltatrix* (Linnaeus)	0	2	84.7	5.9	3.2	6.2	0.0
	Common Eel, *Anguilla rostrata* (Le Sueur)	4	3	77.4	4.6	7.2	10.8	0.0
II	Variegated Minnow, *Cyprinodon variegatus* Lacépède	4	0	95.4	0.9	1.3	2.4	0.0
	Sea Robin, *Prionotus carolinus* (Linnaeus)	4	2	96.4	0.7	1.2	1.7	0.0
	Scup, *Stenotomus chrysops* (Linnaeus)	3	2	95.3	1.4	1.1	2.2	0.0
	Butterfish, *Poronotus triacanthus* (Peck)	3	3	96.2	0.8	1.0	2.0	0.0
III	Toadfish, *Opsanus tau* (Linnaeus)	4	0	99+	rare	rare	rare	0.0
	Tautog, *Tautoga onitis* (Linnaeus)	2	3	99+	few	rare	rare	0.0
	Cunner, *Tautogolabrus adspersus* (Walbaum)	3	1	99+	few	rare	rare	0.0
	Sea Bass, *Centropristes striatus* (Linnaeus)	4	0	99+	few	rare	rare	0.0
	Pipefish, *Syngnathus fuscus* Storer	0	4	99+	few	rare	rare	0.0
	Sand Dab, *Hippoglossoides platessoides* (Fabricius)	2	0	99+	rare	rare	rare	0.0
	Weakfish, *Cynoscion regalis* (Bloch and Schneider)	0	1	99+	rare	rare	rare	0.0
	Sharksucker, *Echeneis naucrates* Linnaeus	0	1	99+	few	rare	rare	0.0
IV	Smooth Dogfish, *Mustelus canis* (Mitchill)	4	4	80.6	5.4	3.4	7.6	3.0
	Spotted Skate, *Raja diaphanes* Mitchill	1	0	83.4	4.6	3.2	6.5	2.3

The first group comprises those in which the proportion of immature erythrocytes is strikingly high, approximately 20 per cent of the total erythrocytes. In the second group the number of immature red cells is much smaller, varying from 3 to 6 per cent. In the third group, the erythrocytes were practically all mature. The fourth group includes the elasmobranchs. These are grouped separately because their erythrocyte picture is quite different from that of the teleosts, resembling more closely conditions found in the cyclostomes, Dipnoi (Jordan and Speidel, 1930, 1931), and urodeles (Dawson, 1930). All stages of maturing erythrocytes, including erythroblasts, were seen and frequently erythrocytes in mitosis were encountered (Maximow, 1923). Summarized results are shown in Table I.

In reading this table it should be borne in mind that the mature erythrocytes of different species vary in the amount of persistent reticulation, the criterion for placing them in Class I being that the reticular material exists as isolated granules or scattered, irregular filaments which may even be branched. In general, the mature cells of the fishes of Group III contain relatively more remnants of the reticulation patterns than those of Groups I and II. Probably not too much significance should be attached to the relative numbers of cells in Classes II, III and IV; the end-points for separating them into these classes are not sharp, and different observers might vary appreciably in their determination of the classes into which such cells should be placed. The figures given are at least indicative of the conditions to be expected, but for quantitative work, where the degree of maturity of the erythrocytes is of importance, reticulation estimates should be made for each species studied.

Discussion

Many of the very active fishes are included in Group I. In general these fish are characterized by their inability to survive for any extended period in restricted quarters, such as the base of the aquarium stands in the Marine Biological Laboratory, even when a moderate inflow of sea water is provided. In the case of the common mackerel this sensitivity to confinement has been found by Hall (1930) to be due, in part at least, to the peculiar adaptation of its respiratory mechanism which permits the animal to secure sufficient oxygen only when it is moving rapidly forward, the ability to respire in a stationary position having been practically lost. The same explanation may hold for some others of this group, menhaden, alewife, summer herring and bluefish, but Hall's experiments apparently have not been extended to include them. In the case of the eel, however, there is no such sensitivity to

confinement; these animals may be kept in tanks for days at a time without exhibiting any signs of respiratory distress.

The fishes of Group II are less active than those of Group I and may successfully be kept in tanks, while most of those in Group III are relatively sluggish, frequently resting quietly in the water. Group IV, comprising the elasmobranchs, need not be discussed in this connection since their blood picture is quite unlike that of teleosts.

Several other sets of data compiled on the blood of teleosts parallel rather closely the present findings on the relative numbers of immature erythrocytes in the circulation. These data are based on determinations of hemoglobin concentration, size of the red corpuscles, numbers of corpuscles per cubic millimeter of blood, lowest oxygen tension at which fish are capable of removing dissolved oxygen from sea water, and the oxygen capacities of the blood.

A comparison of the hemoglobin concentration, measured in milligrams of iron per 100 cc. of blood, has been made of a group of marine fishes by Hall and Gray (1929). Highest concentrations, ranging from 41 to 35.5 mg., were found in the bonito, bull's-eye mackerel, common mackerel, and menhaden. A second group, with concentrations ranging from 23.7 to 27.7 mg., included the cunner, butterfish, scup and sea robin. The remaining fishes of this series vary more widely in this respect, showing concentrations ranging from 21.7 to 11.5 mg. Listed in a descending order these are the rudder fish, puffer, eel, silver hake, goosefish, toadfish and sand-dab. Reference to Table I will show a general agreement between my data and that of Hall and Gray, the most notable exceptions being the eel and cunner. The figures of Root (1931), covering several of the same species of fishes, also show that the high concentration of hemoglobin is associated with small cell-size, high corpuscle count, and high volume per cent of corpuscles.

It is quite evident that all those having high concentrations of hemoglobin are the more active fish. The conditions characterizing their blood presumably impose a greater burden on the erythropoietic centers in that a greater number of corpuscles must be maintained. Any considerable acceleration of the rate of erythrocyte production is rather generally accompanied by the appearance of less mature forms in the blood stream.

No satisfactory explanation has been given for this appearance of immature cells in the circulation. It is held by some that it is due to the limited volume of the erythropoietic organ. With a restricted storage capacity, partially mature cells are forced out to maintain room for the newly-forming cells (Barcroft, 1925). It is probable that some such factor as rate of erythrocyte production is operative in the fishes of Groups I and II.

The underlying stimulus for rapid erythrocyte production is not readily determined. In general the blood picture of active fishes resembles the high-altitude effect obtained in mammals, namely, an increase in total number of red cells and hemoglobin as well as an increase in the number of young cells, reticulocytes. Barcroft has pointed out that the basal principle of the effects of high altitudes is that the blood cell count is a function of the degree of anoxemia. The findings of Hall (1930) on the lowest oxygen tensions at which teleost fishes are capable of removing dissolved oxygen from sea water appear to bear directly on the problem.

Of the fishes studied by Hall the mackerel is unique, being unable to remove dissolved oxygen below a partial pressure of 70 mm. Hg. Other fishes cease respiration at the following tensions: rudderfish, 15.1 mm.; cunner, 14.8 mm.; scup, 13.1 mm.; butterfish, 11.8 mm.; sea robin, 8.5 mm.; fundulus, 8.1 mm.; puffer, 4.7 mm.; sea bass, 3.2 mm.; tautog, 1.6 mm.; and toadfish, 0.0 mm. The eleven species listed may be roughly divided into three groups. One group, represented by the mackerel, removes oxygen only at a relatively high tension, 70 mm. Hg. The second group removes dissolved oxygen between 8 and 15 mm. Hg. pressure, while the third group survives at tensions varying from 0.0 to 4.7 mm. Hg. There is thus a striking correspondence between this grouping of teleosts based on ability to remove dissolved oxygen from sea water and the grouping of the author according to the number of the immature erythrocytes present in the circulation. The cunner, however, is a notable exception.

In an earlier study by Hall (1929) of the scup and toadfish it was found that the scup showed very little change in its oxygen consumption with the oxygen tension between 120 and 40 mm. Hg. pressure but below 30 mm. Hg. pressure a very sharp decline in oxygen consumption occurred and the fishes quickly died whenever the pressure fell below 16 mm. Hg. In the case of the toadfish the oxygen consumption was found to be directly proportional to variations in the oxygen tension between 0 and 118 mm. Hg. Also the toadfish lived for 24 hours with the oxygen tension between 0 and 1 mm. Hg. pressure.

The studies of Hall (1931), Hall and Gray (1929), and Root (1931) indicate that there is a definite correlation between the transportation of oxygen and the environment and habits of the fishes. Adaptations of the respiratory mechanisms are important as shown by Hall (1930, 1931) in his studies of the puffer and mackerel, permitting one form to secure an adequate oxygen supply in a stationary position while the other can do this only when swimming rapidly forward. As already pointed out, sluggish fishes have blood with larger and less

numerous corpuscles, low concentrations of hemoglobin and low oxygen capacities, while active fishes have smaller and more numerous corpuscles, higher concentrations of hemoglobin, and high oxygen capacities—definite adjustments between capacities and requirements. Further evidences of adjustment are also found in the behavior of the hemoglobin at low carbon dioxide tensions. In the presence of 1 mm. of carbon dioxide, a considerably greater tension of oxygen is required to saturate the hemoglobin of the mackerel than that of either the sea robin or toadfish. Root (1931) thinks this may account in part for the great susceptibility of the mackerel to asphyxiation (Hall, 1930).

These morphological and physiological features of the blood, representing adaptations to habits and environment, tend to make the active fish susceptible to variations in the oxygen content of sea water. The high numbers of immature erythrocytes, while probably largely the result of the normal, rapid rate of production of these elements, may also tend to be further increased by stimuli resulting from anoxemia. In other words the margin of safety of active fishes is reduced and the normal condition of their blood may readily pass into a state characteristic of the high-altitude effect found in mammals.

These observations immediately raise the question as to what extent the proportions of immature cells found in the teleosts studied are due to the immediate response of these fishes to varying degrees of asphyxiation caused by methods of transportation and retention in tanks. Studies on the effects of asphyxiation on the menhaden and puffer have been made by Hall, Gray and Lepkovsky (1926) and Hall (1928). These observations showed that asphyxiation produced a definite increase in the concentration of hemoglobin and iron and in the number of red-blood corpuscles, the increase being roughly proportional to the length of time of asphyxiation (19 to 50 minutes). The increased concentration of the blood constituents was explained by a release of water from the blood into the tissues, since the blood volume became diminished during asphyxiation. Iron, however, did not increase in proportion to the loss of water or to the increase in concentration of hemoglobin, but at a faster rate. The spleen showed a decrease in size during asphyxiation and it was suggested that the spleen is a store-house for iron which is released into the blood during asphyxiation.

The observation on the reduction in the size of the spleen may be significant, since in the teleosts this constitutes the major erythropoietic center. In the menhaden, the red-blood cell count rose from 1,988,000 to 3,598,000 per cubic millimeter during 50 minutes confinement in a sealed jar containing 10 liters of water. Since differential counts for degree of persistent reticulation were not made, it is impossible to de-

termine whether the increased number of corpuscles per cubic millimeter was due solely to the withdrawal of water or whether additional and probably less mature erythrocytes had left the spleen to enter the circulation. If erythrocytes do leave the spleen during asphyxiation, as the decrease in its size suggests, it would seem to constitute a possible source of the immature erythrocytes encountered in the circulation.

In the present study the environment of the fishes, especially as regards oxygen tension, was not rigidly controlled. Fish were brought from the traps in the morning and delivered directly to the laboratory in pails of sea water, and many of the more active forms such as mackerel, menhaden, alewife, and summer herring frequently exhibited obvious signs of respiratory distress when received. However, counts made from alewife and mackerel taken directly from a floating "live car" did not differ appreciably from those made on fish delivered by the collecting staff to the laboratory. Since the oxygen tension of the sea water was not determined at the time blood was removed, no statement can be made as to the degree of asphyxiation, if any, to which the fish were subjected.

The prime purpose of this survey was to ascertain the relative homogeneity of the erythrocyte picture in fishes under ordinary laboratory procedures when no particular precautions are taken to insure an optimum environment—conditions such as may prevail when blood is obtained for studies on hemolysis and erythrocyte respiration. If the varying degrees of asphyxiation to which the fish were possibly subjected have modified the blood picture as described in this paper, then such errors are inherent in the data and no correction for them can be made at this time.

Accordingly the variations in the numbers of immature cells either may represent the varying ability of the fishes to respond to the adverse conditions encountered following removal from their natural environment or are evidences of the specific adjustments that have been made between the production of the oxygen-transporting elements, the red-blood cells, and the structural adaptations, the environment, and the habits of the fishes. In fact, these alternatives need not be mutually exclusive. The response to asphyxiation, if proven to occur, may be but an exaggerated picture of conditions normally present in a particular species.

In the elasmobranchs, as in the cyclostomes, Dipnoi, and urodeles, differentiation and multiplication of erythrocytes takes place to a variable extent in the peripheral circulation, and all stages, from their hemoblast progenitors to the completely differentiated cells, may be encountered. Mitosis may also occur in all stages of differentiation, short of

actual maturity. The counts include only cells which contain hemoglobin, the hemoblasts and proerythroblasts being omitted, and are based on large, freshly-caught specimens.

Young dogfish which had been kept in "live cars" for some time showed a higher number of immature cells as well as an increase in the number of mitoses. The blood appeared to be in an actively regenerative phase, probably brought about by the conditions of confinement and diminished food supply. The blood picture of elasmobranchs stands in striking contrast to that of teleosts where the erythrocytes enter the circulation in a much later stage of differentiation and no proliferation by mitosis is observed in the peripheral circulation.

Summary

The results of a study of the blood of the general circulation of twenty species of marine fishes are given. The number of immature erythrocytes present varies widely.

The differential erythrocyte counts were based largely on supravital preparations stained with brilliant cresyl blue. This material was supplemented by dry-fixed smears stained by Wright's method. The reticulation patterns produced by brilliant cresyl blue are discrete structures and more accurate counts can be made on these preparations than on stained smears where the varying degrees of basophilia and polychromasia are used as criteria of immaturity.

The twenty species of fish examined fall naturally into four groups. In Group I, including the mackerel, menhaden, alewife, summer herring, bluefish and common eel, the proportion of immature erythrocytes is high, approximately 20 per cent. In the second group, including the variegated minnow, sea robin, scup, and butterfish the percentage of immature cells is lower, varying between 3 and 6 per cent. In the third group, including toadfish, tautog, cunner, sea bass, pipefish, sand dab, weakfish and sharksucker, practically all the erythrocytes are mature. The fourth group, comprised of elasmobranchs, was treated separately since in these forms erythrocytes are continuously differentiated in the blood stream from primitive cells and may also proliferate mitotically in this location. The number of immature red cells in these fish is also great.

The varying blood pictures appear to represent the result of specific adaptations of fishes to such interrelated factors as their type of external respiratory mechanism, the efficiency of their oxygen-transporting system, their oxygen requirements and the oxygen tensions of their environment. In Group I the general blood picture is suggestive of the high-altitude effect observed in mammals.

Many of the fishes with high counts of immature erythrocytes quickly exhibit signs of asphyxiation when removed from their natural habitat and kept in tanks. Hall has noted that during asphyxiation the spleen of fishes decreases in volume. This may be correlated with the entrance of immature erythrocytes into the general circulation. Accordingly, it is possible that the figures presented in this paper are not exactly representative of the condition of the blood when such fish are undisturbed in their natural environment.

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ON THE RESPIRATORY FUNCTION OF THE BLOOD OF THE PORPOISE

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The capture of two porpoises, *Phocæna phocæna* Linn., in the fish trap operated by the Marine Biological Laboratory has afforded an opportunity to obtain data which have not been available before on the conditions of equilibrium between the blood of a cetacean and the respiratory gases. The mammals of this order are so strikingly modified morphologically in adaptation to their aquatic life that an examination of the physico-chemical properties of the respiratory fluids is attended with some interest. While the results recorded in the present paper require confirmation and extension in many details, the general picture of the physico-chemical system involved in transporting the respiratory gases is sufficiently definite to warrant presentation in the present provisional form.

The porpoises were brought from the trap on May 14 and placed in a large floating crate at the laboratory. One died on the following day and was used to determine the proper approach for cardiac puncture. The other lived until May 18, when it was killed in the course of experiment. During this period it lay in the water rather quietly and did not struggle when held. On May 17 fifty cubic centimeters of blood was drawn by puncture of the left ventricle, the needle being inserted one inch behind and above the caudal edge of the left flipper. The porpoise did not struggle. A 10 cc. portion of this blood was preserved under oil, for the determination of the carbon dioxide content of arterial blood. The remainder was used for the determination of the shape of the oxygen and carbon dioxide dissociation curves. Coagulation was prevented by the use of dry sodium oxalate. The blood drawn at this time will be designated *Specimen A.*

The following day an unsuccessful attempt was made to measure the blood volume by the vital red method. Twenty cubic centimeters of blood were drawn from the heart with some difficulty and it appeared that both right and left ventricles had been pierced as a portion of the sample was not fully oxygenated. This sample was oxalated and used for the determination of oxygen capacity and cell volume and was designated *Specimen B.*

[1] Contribution No. 4.

Immediately after drawing Specimen *B*, the porpoise went into asphyxial convulsions, evidently as the result of cardiac failure. The thorax was opened and 50 cc. of blood were drawn directly from the ventricle. This sample was designated as *Specimen C* and was used for determining the effect of hydrogen ion concentration upon the position of the oxygen dissociation curve.

Methods

Oxygen and carbon dioxide contents were determined by using the Van Slyke constant volume analyzer. The gas mixtures were analyzed with a Haldane apparatus after equilibration and the pressures calculated by means of the equation developed by Bock, Field, and Adair (see Henderson, 1928, p. 384). The blood was equilibrated with the gas

Table I

Oxygen Capacity and Volume of Erythrocytes in the Blood of Aquatic Mammals

Species	Porpoise Phocæna phocæna			Dolphin Tursiops tursio	Sperm Whale Physeter macro-cephalus	Sea Lion Eume-topias stelleri
Specimen	A	B	C			
Oxygen capacity, vol. per cent	22.15 22.20	19.8 19.7	20.44 20.34			19.8
Oxygen combined, vol. per cent	21.78	19.35	19.99	31.8	29.09	19.4
Volume of cells in 1 cc. blood, cc	0.35	0.35	0.36	0.517		0.29
Oxygen combined in 100 cc. cells, vol. per cent	62.2	55.4	55.6	61.5		67

mixtures at 38°, preserved in blood-sampling tubes over mercury, and promptly analyzed. The original specimens of blood were preserved in the refrigerator until used, and all measurements were made within twenty-four hours of the time the specimens were secured.

The Oxygen Capacity of the Blood and of the Erythrocytes

Table I contains the data obtained from the three specimens of blood we have examined, together with results reported by Jolyet (1902) for the dolphin and sperm whale, and by Florkin and Redfield (1931) for the sea lion. The oxygen capacities we obtained, like those observed in the sea lion, are of the same magnitude as those commonly found in terrestrial mammals. They contrast strongly with the higher values recorded by Jolyet and Sellier (1896–97) for the porpoise (30.9 volumes

per cent), by Jolyet (1902) for the dolphin and sperm whale, and with the values of 42.5 and 45.1 volumes per cent reported by Sudzuki (1924) for the blood of porpoises. Whether these differences are characteristic of the different species in question, or are due to accidental causes, such as a possible anæmic condition of the individuals yielding the lower values, or to experimental errors, cannot be decided until more data are available. We were impressed with the rapidity with which the corpuscles settled out when the porpoise blood was allowed to stand and believe that errors large enough to account for the differences reported for the different species might readily arise from this cause were it not guarded against. The observations are all in agreement in showing that a given volume of the corpuscles of these aquatic mammals combines more oxygen than does an equal volume of

TABLE II

Data on carbon dioxide equilibrium in porpoise blood. Temperature, 38° C.

	Oxygen pressure	Carbon dioxide pressure	Carbon dioxide content
	mm. Hg	*mm. Hg*	*vol. per cent*
Specimen A	150 ca.	30.7	35.8
	150 ca.	51.3	46.0
			46.2
Specimen C	150 ca.	41.0	25.6
			25.5
	150 ca.	51.1	29.7
			29.0
	1.4	51.8	32.45
			32.30

corpuscles of the terrestrial mammals, in which 100 cc. of corpuscles have an oxygen capacity of about 45 cc.

THE CARBON DIOXIDE DISSOCIATION CURVE

Table II records the data obtained by equilibrating Specimens *A* and *C* with carbon dioxide. From these data the carbon dioxide dissociation curves illustrated in Fig. 1 have been constructed by making use of the approximation (Henderson, Bock, Dill, and Edwards, 1930):

$$\log T = A \log (pCO_2) + B,$$

in which T is the total carbon dioxide content of the blood, A and B are constants, and pCO_2 is the carbon dioxide pressure. The curve constructed from data on Specimen A does not differ greatly from that of normal man, though the carbon dioxide combined at any pressure is somewhat less. The curve for Specimen C represents blood drawn following death from asphyxia, and the combined carbon dioxide is consequently less at each carbon dioxide pressure.

These curves may be used for comparing the buffer action of porpoise blood with that of human blood. Henderson, Bock, Dill, and Edwards (1930) have prepared a nomogram showing the relation between the slope of the carbon dioxide dissociation curve, its height, and the hemoglobin content of human blood based on an examination of

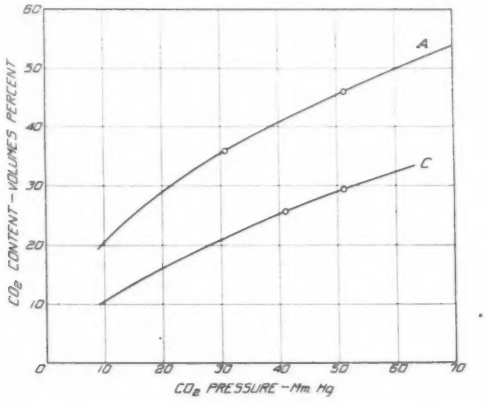

FIG. 1. Carbon dioxide curve of blood of porpoise. Upper curve, Specimen *A*. Lower curve, Specimen *C*. For data see Table II.

some 117 specimens. In Table III are recorded the values of *Hb*, the hemoglobin content as measured by oxygen capacity; T_{40}, the carbon dioxide content at 40 mm. carbon dioxide pressure; and Δ, the difference in carbon dioxide content at 30 and 60 mm. carbon dioxide pressure as deduced from the curves and data describing Specimens A and C. In this table are also recorded the values of Δ characteristic of human blood having equivalent values for *Hb* and T_{40}, taken from the nomogram of these authors. Both specimens of porpoise blood have slightly higher values of Δ than does comparable human blood. The differences, however, are not greater than might be accounted for by experimental error. Comparable data for the blood of the sea lion, from the measurements of Florkin and Redfield (1931), and mean

values for dog blood given by Dill, Edwards, Florkin, and Campbell (1932) are included in the table. The comparison serves to emphasize the similarity of the bloods when regarded as systems for transporting carbon dioxide.

Table II contains measurements of the carbon dioxide content of a sample of Specimen *C* equilibrated with carbon dioxide in the virtual absence of oxygen. Comparing the results with the value obtained from the curve in Fig. 1 for the same carbon dioxide pressure, it appears that the reduced blood combines about 2.86 volumes per cent more carbon dioxide than does oxygenated blood. This effect is similar to that observed in other mammalian bloods but is rather smaller than usual for blood of comparable oxygen capacity.

TABLE III

Comparison of Buffer Value of Oxygenated Porpoise and Sea Lion Blood with that of Man

Species	*Hb* Oxygen capacity	T_{40} CO_2 content at pCO_2 = 40 mm.	Δ observed	$T_{60} - T_{20}$ calculated from human blood	Ratio
	milliequiv. per liter	*milliequiv. per liter*	*milliequiv. per liter*	*milliequiv. per liter*	
Porpoise					
Specimen A	9.73	18.18	6.52	5.80	1.12
Specimen C	8.93	11.22	5.12	4.70	1.09
Sea Lion	8.88	16.02	5.32	5.30	1.00
Dog (Mean)	9.7	18.1	5.78	5.76	1.00

THE OXYGEN EQUILIBRIUM

Data on the equilibrium of Specimen *A* with oxygen are presented in Table IV. In order to fit the measurements, the oxygen dissociation curve shown in Fig. 2 was drawn using Hill's equation

$$y = \frac{Kx^n}{1 + Kx^n} \times 100,$$

in which y is the percentage saturation of the hemoglobin, x the oxygen pressure, $K = 0.00102$ and $n = 2.1$. The carbon dioxide pressure of these samples was about 46.5, corresponding to a carbon dioxide content of 44 volumes per cent. Taking $pK = 6.14$, yields a value for pH of 7.29. The values for n which have been used to characterize the blood of man vary from 2.2 to 2.5. The shape of the oxygen dissociation curve of the porpoise so far as it is characterized by n does not differ markedly from human blood.

The portion of Specimen *A* which was collected under oil had a carbon dioxide content of 45 volumes per cent, corresponding to a carbon dioxide pressure of 49 mm. and a pH of 7.27. Consequently the oxygen dissociation curve in Fig. 2 represents approximately the conditions characteristic of the blood *in vivo*, if one neglects the change in oxygen

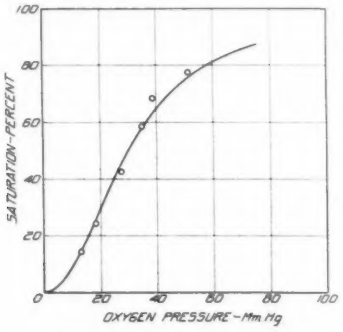

FIG. 2. Oxygen dissociation curve of blood of porpoise at a carbon dioxide pressure of approximately 46 mm.—equivalent to that of arterial blood. For data see Table IV.

equilibrium, due to the increased carbon dioxide content of the venous blood.

THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE OXYGEN DISSOCIATION CURVE

Table V contains data for the oxygen equilibrium of samples of Specimen *C* obtained at a variety of carbon dioxide pressures. Using

TABLE IV

Data on the equilibrium of oxygen with porpoise blood, Specimen A.
Temperature, 38° C.

Carbon dioxide pressure	Oxygen pressure	Oxygen content	Oxygen dissolved	Oxygen as oxyhemoglobin	Saturation
mm. Hg	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*
46.5	13.0	3.14	0.03	3.11	14.3
46.4	18.1	5.30	0.05	5.25	24.1
46.6	27.5	9.37	0.07	9.28	42.5
47.2	34.9	12.86	0.09	12.77	58.6
38.7	38.7	14.95	0.12	14.83	68.1
42.7	51.4	16.98	0.15	16.83	77.3
air	air	22.18	0.40	21.78	100.0

Hill's equation and taking the value of $n = 2.1$, the values of K, and of p_{50} (the oxygen tension at which the blood would be half saturated with oxygen) have been calculated. As an empirical procedure this practice involves only the assumption that the form of the oxygen dissociation curve, determined by the value of n, remains unchanged at various carbon dioxide tensions. This assumption is amply justified by the behavior of other mammalian hemoglobins. The hydrogen ion concentration of the various samples have also been calculated with the aid of the

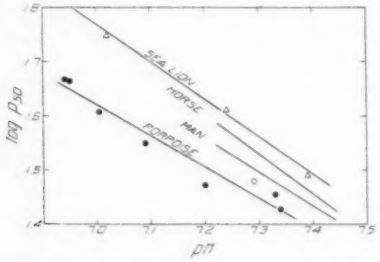

FIG. 3. Relation of p_{50}, the oxygen tension at which blood is half saturated, and the pH of the serum of whole blood. For data see Table V and references in text. Hollow circle, porpoise Specimen *A*. Solid circles, porpoise Specimen *C*.

carbon dioxide dissociation curve for Specimen *C* in Fig. 1. From these values the relation between p_{50} and pH shown in Fig. 3 has been plotted.

For precise comparison of the properties of hemoglobin in the blood of different animals the properties should be referred to the pH of the cell contents. Unfortunately our studies did not include the measurements necessary to enable us to calculate this value. We have conse-

TABLE V

Data on the equilibrium of oxygen with porpoise blood (Specimen C) at different carbon dioxide tensions. Temperature, 38° C.

Carbon dioxide pressure	Oxygen pressure	Saturation	log p_{50} calc.	pH
mm. Hg	*mm. Hg*	*per cent*		
9.86	34.4	62.7	1.428	7.34
10.5	23.5	40.0	1.455	7.33
21.4	18.4	26.7	1.473	7.20
39.7	27.1	36.3	1.550	7.09
52.5	45.1	56.5	1.607	7.04
87.6	38.9	41.2	1.664	6.95
92.0	54.4	58.3	1.666	6.94

quently compared the values of p_{50} obtained at different carbon dioxide pressures with those characterizing other bloods when the pH of the serum of the whole blood was the same. This comparison is included in Fig. 3, the data for the horse and for man being taken from the nomograms given by Henderson (1928); that for the sea lion is calculated from the data of Florkin and Redfield (1931). When compared on this basis the hemoglobin of the porpoise does not differ strikingly from that of other mammals and does not differ from the blood of normal man much more than the blood of men differ from one another.

Discussion

The preceding observations all tend to indicate that the general physico-chemical properties of the blood of the porpoise are very similar to those of the terrestrial mammals. Even in the strictly quantitative aspects of the various properties of the blood one can detect very little variation from the mean conditions observed in human blood. The increased concentration of hemoglobin in the corpuscles of the cetaceans and the sea lion is perhaps the only clear-cut condition which might be regarded as an adaptation to the more rigorous respiratory requirements of aquatic life.

Unfortunately, our attempts to determine the blood volume of the porpoise were unsuccessful. This relation is, in our opinion, the most important aspect of the respiratory mechanism which remains to be examined. One cannot help being struck by the richness of the muscular tissue in hemoglobin. It seems probable that in this condition, and perhaps in other modifications of the chemical situation in the muscles, adaptations to aquatic life may be found.

It should be pointed out that the porpoise is not in the habit of remaining submerged for long periods of time when compared to the larger Cetacea and certain other aquatic mammals. Parker (1932) records that in a porpoise confined in a large tank, the average respiratory interval was 15.48 seconds. The shortest interval observed was 6.5 seconds, the longest 31.7 seconds. These are not strikingly greater than the intervals of inspiration in man and might easily be maintained, at least temporarily, by voluntary effort in his case. When feeding in the open sea, one might expect the intervals to be greater and Jolyet and Kükenthal (quoted by Parker) note that dolphins may remain under water much longer, Jolyet setting the maximum at about fifteen minutes. The general testimony of whalers indicates that the larger Cetacea are in the habit of remaining submerged for much longer periods. Whether more distinct adaptations of the physico-chemical mechanisms of the blood will be found in such forms remains to be seen.

SUMMARY

The properties of the blood of the porpoise, *Phocæna phocæna*, considered as a system for the transport of oxygen and carbon dioxide, have been examined.

It does not appear that the morphological changes adapting these animals for aquatic life are accompanied by significant modifications in the physico-chemical properties of the blood.

The only striking characteristic of porpoise blood, when compared with that of terrestrial mammals, is an increased concentration of hemoglobin in the corpuscles.

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THE EXTERNAL FIBRILLAR SYSTEM OF EUPLOTES WITH NOTES ON THE NEUROMOTOR APPARATUS

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The nature of the external fibrillar system of ciliate Protozoa (" Silberliniensystem " of Klein, 1926*a*, 1926*b*) and its relation to the better-known neuromotor apparatus as first described by Sharp (1914) for *Diplodinium* has been the subject of considerable research of late years. Marked differences of opinion obtain as to the function of both these types of fibers and even their structure has not been agreed upon.

With the hope of solving some of the problems concerned, *Euplotes patella* was chosen for the subject of this investigation chiefly because its neuromotor system is probably better known than that of any other ciliate. Its description given by Yocum (1918) and the demonstration of its function by Taylor (1920) serve as two of the chief milestones toward an understanding of the fibrillar makeup of ciliates.

The description of the external fibrillar network in *E. patella* was originally undertaken by Mr. Samuel Yabroff in 1925 at the University of California. Later he gave up this work when he entered medical school and turned the problem over to me with the statement that he was no longer interested in it.

This work was aided by a research grant from the Graduate School Research Fund of the University of Minnesota.

TECHNIQUE

Euplotes for this study was cultivated in mass on wheat and timothy hay as described by Turner (1930). The methods of fixation and staining or silver impregnation are outlined below.

For the External Fibrillar System

Silver method of Klein (1926*b*). This gave fair results but drying caused too much distortion. Fixing first in osmic vapor helped some.

Brown's (1930) method of using thionin after Mann's fixation did not show the fibrils at all. Like Liebermann's (1929) nigrosin method it is excellent for *Paramecium* but useless for *Euplotes*.

Silver-gelatin method of Chatton and Lwoff (1930). A few specimens that are around the border of the main drop of gelatin show the network very well, but it is difficult to get just the proper amount of gelatin on the slide.

Formol-osmium-toluidin blue method of Gelei (1927) was useless as no differentiation could be obtained.

The technique which consistently gave the best results for whole preparations was a variation of Klein's and Gelei and Horváth's (1931) methods: Place a small drop of material containing the organisms on a clean slide previously smeared with a light coat of egg albumen. Draw off excess water with a micro-pipette. Fix with osmic acid vapor for about three seconds. This may be easily done by simply inverting the slide over a bottle containing 2 per cent osmic acid. Place slide in a cool breeze (4° to 15° C.) until the material is nearly but not quite dry in the center. Flood gently with two or three drops of 2 per cent silver nitrate. A few of the organisms will be lost but most of them will be caught in the albumen. After 4 to 8 minutes in the silver nitrate, pour off the excess and place the slide in a white dish, or glass dish over white paper, containing enough distilled water to cover the slide. Place in the sun in a cool place and watch the progress of the reduction by occasional examinations with the microscope. When the desired depth has been obtained, wash thoroughly in distilled water, dehydrate, and mount. If slower reduction is desired, exposure to bright sky but not to the sun is efficacious. This method gives strikingly clear-cut results.

Aside from the silver impregnation methods the only way found of demonstrating the network with any degree of clarity and completeness is by drying specimens on a clean slide and staining with thionin. This is done by simply flooding the specimens with a 0.5 per cent aqueous solution of the stain for a few seconds, rinse and dehydrate as rapidly as possible, clear and mount. While this method gives essentially the same picture as the silver methods, less contrast is obtained by it.

Bresslau's (1921) method was used but did not prove satisfactory for *E. patella* although it is good for *Paramecium*.

For sectioning, the only method worth mentioning favorably out of the many tried is that of Gelei and Horváth (1931), using the maximum strength and time for the silver nitrate. After clearing, some of the material was mounted whole and the rest embedded and sectioned 3 to 15 microns thick. These sections were valuable chiefly in showing the location of the fibers in the protoplasm.

Although I have tried many times to repeat the method used by Yabroff (1928) on *Euplotes*, I have consistently failed to get satisfactory results. Others in private correspondence have reported similar failures, but Pickard (1927) reports conspicuous success with it on *Boveria*. In my opinion it is a capricious technique but one which has possibilities and with good fortune and the proper twist of the wrist will be found valuable. *Intra vitam* stains used were neutral red, Janus

green B, dahlia violet, methylene blue, dilute hæmatoxylin, and thionin. All were tried in concentrations of from 1–1,000 to 1–100,000. Neutral red at about 1–10,000 gave the best results.

For the Neuromotor Apparatus

Zenker's and the picro-mercuric fluid of Yocum, followed by Mallory's connective tissue stain as used by Sharp. Sections 2, 4, 6, 8, 10, 15, and 20 microns thick were made in addition to whole mounts. Beautiful preparations were obtained with this stain following either fixative, but the colors fade. In the thicker sections and the whole mounts the general picture is seen fairly well, while the details may be better studied in the thinner sections. Whole mounts were usually fixed in picro-mercuric as the high alcohol-ether content caused the organisms to adhere to the slides much more readily than did Zenker's.

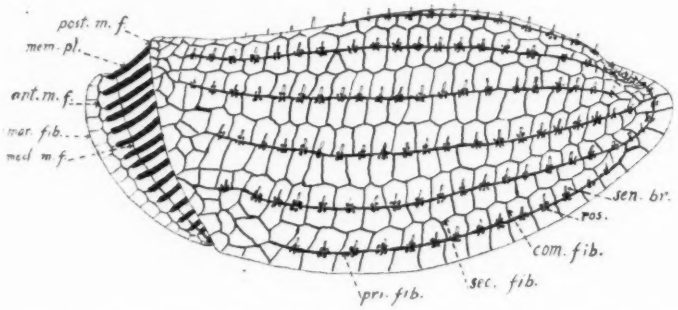


FIG. 1. Dorso-lateral view of external fibrillar system of *Euplotes patella*. Rosettes and sensory bristles from neutral red specimen, the rest from silvered specimen. *Ant. m. f.*, anterior membranelle fibril; *com. fib.*, commissural fibril; *mar. fib.*, marginal fibril; *med. m. f.*, median membranelle fibril; *mem. pl.*, membranelle plate; *post. m. f.*, posterior membranelle fibril; *pri. fib.*, primary fibril; *ros.*, rosette; *sec. fib.*, secondary fibril; *sen. br.*, sensory bristle.

Delafield's, Heidenhain's, Apáthy's and Dobel's hæmatoxylins were used following Schaudinn's, both hot and cold, and Flemming's strong fixing fluids. Schaudinn's followed by Heidenhain's gave the best results with whole mounts while Flemming's and Heidenhain's proved the most satisfactory for sections.

Many other methods were tried without conspicuous success.

Intra vitam stains as mentioned above for the external fibers were used. Neutral red 1–20,000 allowed to act for 1 to 3 hours, and thionin of about the same dilution were found most useful.

The External Fibrillar Network

The convex dorsal surface of *Euplotes patella* shows seven to nine longitudinal rows of granules arranged in little rosettes (Fig. 1). From the center of each rosette a bristle protrudes externally. Griffin (1910) states that in *E. worcesteri* sensory bristles protrude from these rosettes, and recently Jacobson (1931), using a silver technique, describes and figures a central bristle protruding from a ring instead of a rosette, in *E. patella*.

I have observed these bristles not only in material impregnated with silver, but also in living specimens stained *intra vitam* with neutral red. Each bristle is about two microns long and perhaps one-tenth micron

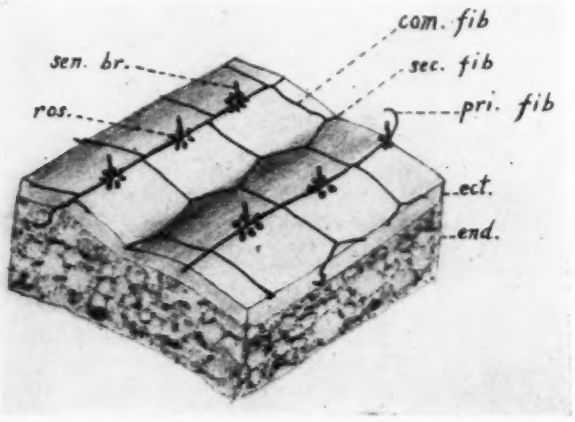


Fig. 2. Enlarged section of dorsal body wall to show position of fibrils in ectoplasm. Note several places where fibrils were not cut off clean with the section. *Ect.*, ectoplasm; *end.*, endoplasm; other labels as in Figure 1.

thick. Its base is surrounded by four to eight elongate granules arranged radially (Fig. 2). In silver preparations the granules of the rosette are usually clumped into a single blob as seen in the photomicrograph (Pls. I, II, and III). That the bristles are not cilia is evident from the fact that they are not vibratile, although it is quite possible they have been evolved from ancestral cilia.

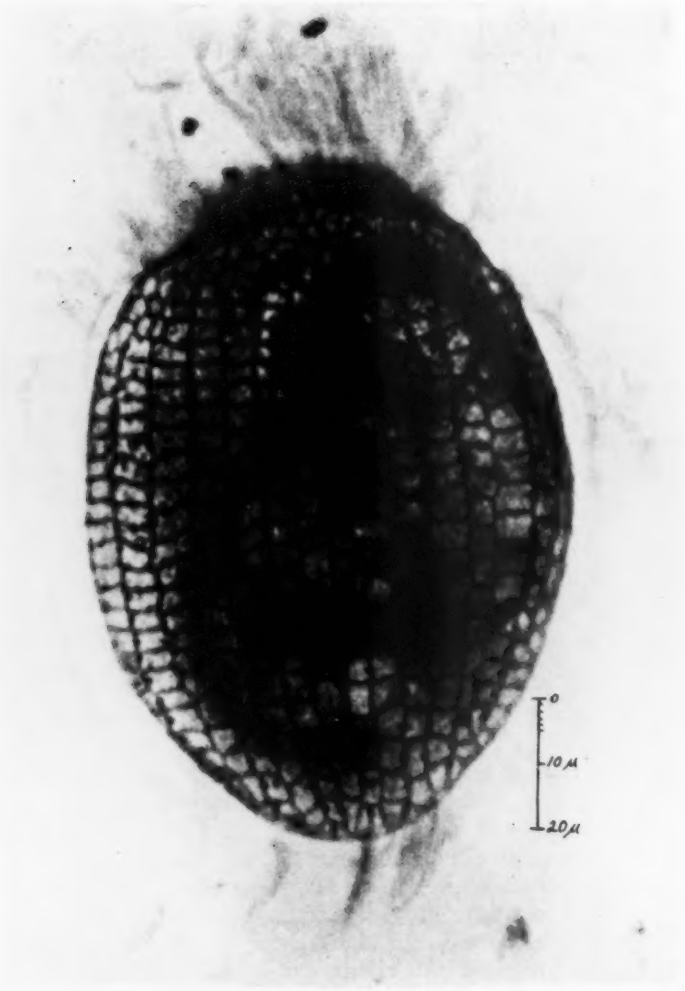
Connecting up the rosettes are seven to nine (usually nine) longitudinal fibrils. These I have called the *primary fibrils* as they are the heaviest and most easily seen, and because they are associated with the bristles and rosettes. Halfway between the primaries and extending parallel to them are the *secondary fibrils* which are only slightly less

regular than the primaries. In addition to these there are *commissural fibrils* extending across from the primaries to the secondaries causing the secondaries to appear as though pulled slightly out of line. This creates a veritable network or latticework which is remarkably constant in appearance, the squares of which average about four microns across (Pls. I and II). All these structures have been observed in neutral red material demobilized with osmic acid vapor. This reminds one of Fig. *F* in Pickard's (1927) paper on *Boveria*. Myonemes, however, have no place in *Euplotes* as the cortex is rigid.

Anterially, the network is connected with the *posterior membranelle fiber* (Fig. 1) at the edge of the dorsal cortex. Both primaries and secondaries are fastened to this fibril either directly or by a more or less irregular anastomosis of the fibrils.

Anterior to the dorsal cuirass is the collar in whose surface is seen an extension of the fibrillar system, part of which was described by Yocum. Extending anterio-ventrally is the row of basal plates of the membranelles (Fig. 1). These are attached to the *posterior membranelle fibril* at the proximal end and extend about two-thirds the distance to the margin of the lip where they connect with the *anterior membranelle fibril* by means of short commissures that extend from their tips. This anterior membranelle fibril is the "membranelle fiber" (anterior cytostomal fiber) of Yocum's neuromotor apparatus. This in turn is connected to the marginal fibril by short commissures. Linking up the basal plates of the membranelles is still another fibril about two-thirds the distance to their anterior tips. This *median membranelle fibril* is seen only in the clearest preparations as it is easily blocked out by the heavy impregnation of the membranelle plates. In all probability it was part of this collar equipment that Yocum saw and considered sensory structures.

On the ventral surface of *E. patella* the network, instead of being composed of a cross-hatching of lines, appears less regular and reminds one of badly treated chicken wire (Plate III). Hexagons form a rather prominent part of the network while rectangles, squares, etc., are not infrequent. The pattern, however, as seen in any of the individuals is surprisingly constant and characteristic. For example, the hexagons of the ventral surface of the oral lip, the long slender rectangles extending posterior to the tip of the peristomal field, and the squares of the lateral phlanges are always present. The squares of the lateral phlanges are made by fibrils similar to those of the dorsal surface, the marginal fiber being a secondary and the submarginal being a primary fibril provided with rosettes and sensory bristles as are those of the dorsal surface.

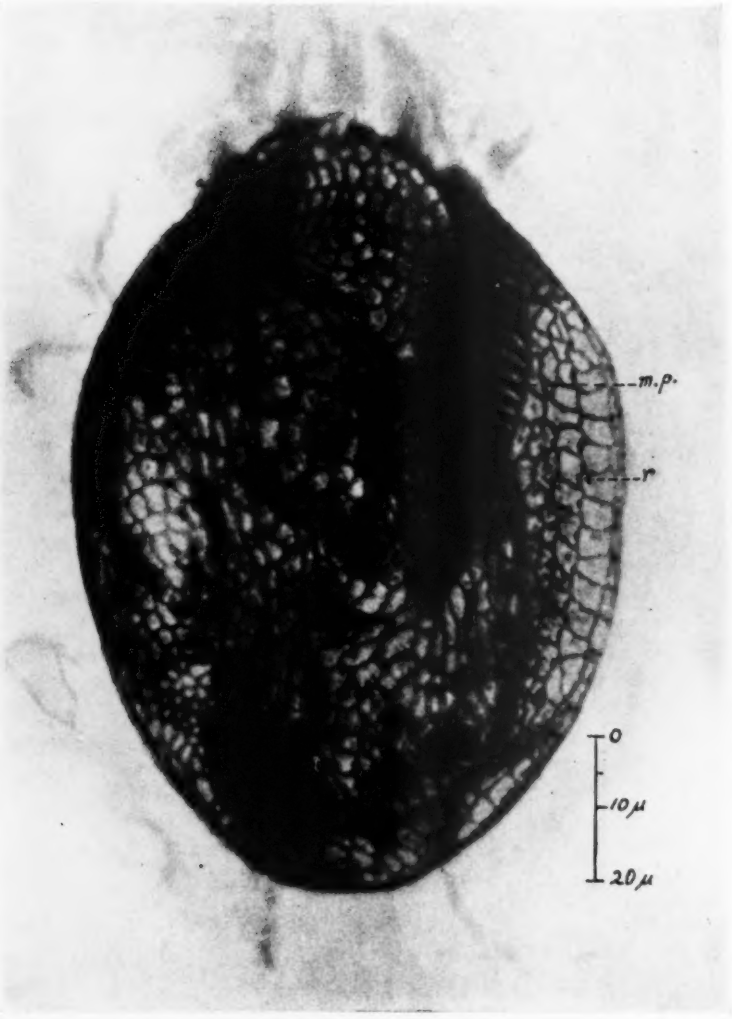


EXPLANATION OF PLATE I

Photomicrograph of dorsal network of *E. patella*. Silver nitrate preparation.

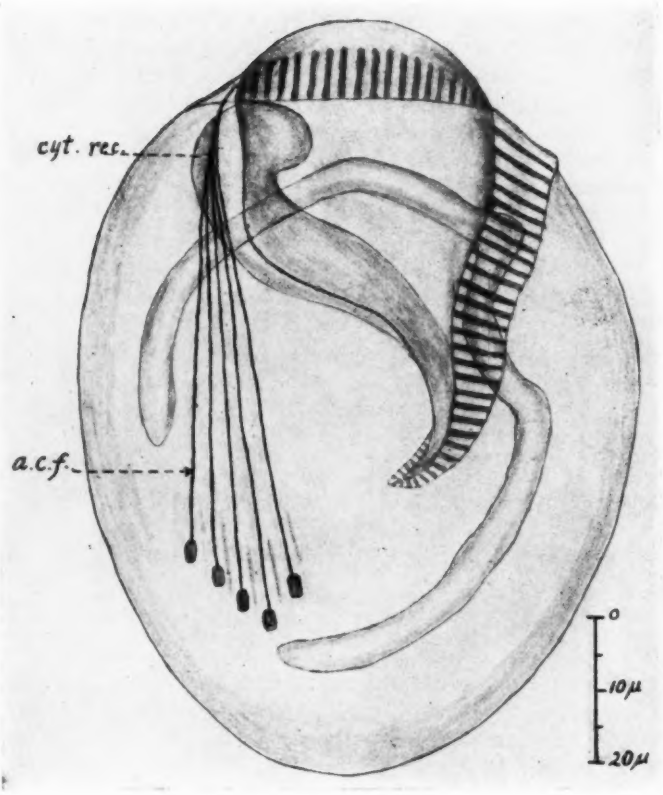
EXPLANATION OF PLATE II

Photomicrograph of dorsal network of another specimen. Rosettes can be seen clearly near the sides as single blobs. Silver nitrate preparation.



EXPLANATION OF PLATE III

Photomicrograph of ventral network of *E. patella*. *m. p.*, membranelle plate in lateral peristomal field; *r.*, rosette. Silver nitrate preparation.



EXPLANATION OF PLATE IV

Camera lucida drawing, ventral view, of *E. patella* showing the anal cirri fibers of the neuromotor apparatus passing directly into the collar, unbroken by a motorium. All other cirri and fibers omitted from the drawing. *A. c. f.*, anal cirri fibers; *cyt. rec.*, cytostomal recess. Schaudinn's and Heidenhain's hæmatoxylin.

The basal plates of the membranelles located in the left margin of the peristome (Plate III and Fig. 3) are deeply impregnated by the silver and appear like ties of a railroad track. Extending down the center of the "roadbed" like a loosely strung wire is the fibril which connects basal plate to basal plate—a continuation of the median membranelle fibril. Bordering each side of the peristomal field of membranelles and in direct contact with the ends of the basal plates are the two other membranelle fibrils.

Sectioned material shows the fibrils of both dorsal and ventral networks to be immediately under the pellicle and in contact with it. They appear round in cross-section and the fibrous nature is disclosed by the fact that frequently they are not cut off clean with the section but protrude from the edge like loose threads at the end of a frayed piece of cloth (Fig. 2).

FIG. 3. Camera lucida sketch of a portion of the membranelle field at about point *m. p.* in Plate III, but taken from another specimen. *A. m. f.*, anterior membranelle fibril; *m. m. f.*, median membranelle fibril; *p. m. f.*, posterior membranelle fibril, all continued down from collar region. Silver nitrate preparation.

It is worthy of note that the apparent heavy nature of the fibrils is due to the impregnation of silver, as in material stained *intra vitam* the fibrils appear as exceedingly delicate strands.

NEUROMOTOR APPARATUS

Regarding the neuromotor apparatus, my observations tend to confirm those of Yocum except in the matter of the motorium. The fibers from the bases of all cirri except the anals were found to extend considerably farther through the cytoplasm than indicated by Yocum, but this is a matter of no special significance.

After examining literally hundreds of specimens both entire and in serial sections 2, 3, 4, 6, 8, 10, 15 and 20 microns thick, using all the

techniques suggested for its demonstration, I am forced to the conclusion that there is no motorium in *Euplotes patella* as a separate and distinct body. I have never been able to see it even in specimens where the other neuromotor elements are exceptionally clear. It is quite evident in many of my preparations, both whole mount and sectioned, that the five anal cirri fibers converge, join to form one fiber and extend on into the collar without a break (Plate IV). Here the single fiber turns and runs along the anterior (outer) edge of the "collar" membranelles thence along the inner (median) edge of the "lapel" membranelles. Yocum shows the five fibers attached to the left end of the motorium (his Plate XV, Fig. 9) while the fiber to the membranelles is attached to the right end of the motorium. This makes quite a break in the continuity of the system. It is easily seen in my material that there is absolutely no jog or break in the fibers from the time they leave the cirri until they reach the membranelles. Furthermore, there is no branch line running off to any body that might be considered an attached motorium, nor is there a body which is nearby and consistently present which might be a motorium.

It is quite possible that any one of several structures in this region might be mistaken for a motorium, namely, the basal plate of one of the two or three cirri located there, groups of granules such as are found scattered through the cytoplasm, or a fold of the ectoplasm in the rather thin right peristomal phlange. Professor Yocum has kindly sent me one of his slides showing what he considers the motorium on two marked specimens. Although it is hardly just to judge from two specimens, it seems to me that the darkened area which he interprets as the motorium is a wrinkle in the ectoplasm overlying the fibers on the ventral surface. I find similar structures in my own preparations if the material is fixed on slides but not in material killed in bulk. The logical explanation of this is seen in the anatomy of the organism. In the region concerned the roof of the cytostome contains a peculiar pocket, the cytostomal recess (Plate IV). When the animal is flattened by being fixed on the slide the peristomal phlange in this region is folded by being pressed against this irregularity. Such a fold occurring regularly at this point could easily be interpreted as a motorium. Yocum's Figure 9, Plate XV, can be explained in this light if the part of the membranelle fiber before it reaches the collar be considered a continuation of the edge of the cytostomal recess. However, his Fig. 5, Plate XIV, is difficult to explain on these grounds. Such a structure might have resulted from a collection of endoplasmic granules as so frequently occurs in this species. My interpretations must be based on the fact that in my material no such body is present as that which he labels "motorium,"

and the membranelle fiber, instead of giving off two roots to this body, is clearly and directly continuous with the five anal cirri fibers. Serial sections stained with Mallory's triple and with the hæmatoxylins support the camera lucida drawing of the whole mount shown in Plate IV.

Rees (1930) concludes that in *Diplodinium* the object called a motorium by Sharp (1914) is only a fold of the ectoplasm, and the careful investigation that he made is quite convincing. The "motorium" of *Euplotes patella* is probably a similar structure.

Discussion

The composite picture of *Euplotes patella* shows an amazing array of fibrils which link up the organelles. This consists of a superficial network linking up bristles that protrude from the surface, and a deeper set which is associated with the motor organelles. The two are connected in the anterior region.

This picture in itself strongly suggests a sensory apparatus and a motor apparatus linked together to form an integrated coördinating system.

Klein (1926*a*) was the first to give us a detailed picture of the external fibrillar network in a ciliate. Since then he has described the "Silberliniensystem" of a number of ciliates, some of which are beautifully illustrated, some very poorly. The latter includes a distorted and almost unrecognizable *Euplotes* of undetermined species (Klein, 1928). In this paper he states that the new peristomal field (as seen in the new complex of fibers) appears before any other sign of division. Turner (1930) has shown, however, that the macronucleus is the first structure to show signs of an approaching division. In another paper Klein (1926*b*) pictures the rosettes of *E. harpa* as single granules and calls them "Basalkörner." In this paper he proposes the idea that the fibrillar network is the real coördinating system in ciliates, and in *Euplotes* it is the real neuromotor system while the system described by Yocum is contractile in function. According to him the microdissection experiments of Taylor were confused. Claiming it to be a primitive nervous system endowed with both motor and sensory functions, Klein (1929) further ascribes to the "Silberliniensystem" the power of initiating division, controlling morphogenesis and to some extent inheritance. His evidence for all this is not completely convincing.

The German workers generally consider the "classical" neuromotor apparatus as a contractile or supportive structure rather than conductile. In *E. patella* the only possible function of contractile elements would be for the operation of the cirri or membranelles, and as these motor organelles are actually groups of cilia, one would not expect them to be

operated by such contractile fibers on the basis of what is known of ciliary movement. Taylor's observations show also that the anal cirri continue to function even after the fibers are cut, a fact that rather demolishes the notion that the contraction of the fibers mechanically operates the cirri. Again, the fact that no movement can be detected in the fibers when the cirri are beating is further evidence against this idea.

Without a motorium the neuromotor system of *E. patella* is still established as a definite apparatus. A coördinating *center* is a nice concept, but one which is not indispensible to a neuromotor apparatus. Taylor's results are as significant without a motorium as with, when one considers the fibers themselves as the coördinating mechanism; the continuity of the fibers from the membranelles to the anal cirri maintaining the coördination.

The function of the external fibrillar network is less well established. In *E. patella* where the cortex is so strong and unyielding it is difficult to believe that a network so delicate as that seen in living specimens could add much to the rigidity of the body. From purely morphological evidence it appears that the network is sensory in nature. It is just under the pellicle where one would expect such a system, and it connects up all the bristles which appear to be sensory elements. The joining of this system to the neuromotor fibers makes a complete conductor system—sensory and motor—that seems not only adequate to interpret the structures seen, but also to explain what is known of the reactions of the organism.

In the more primitive ciliates with cilia distributed over the body, the "silver lines" may well be both sensory and motor in function, forming a kind of primitive coördinating apparatus which controls the action of the cilia in response to stimuli received. In *Euplotes,* which is one of the most highly organized of all the Protozoa, it is not surprising to find the conductor system more or less divided up into sensory and motor departments comparable in a way to the more highly specialized members of the Metazoa.

Summary

1. The external fibrillar system or "Silberliniensystem" of *Euplotes patella* is described as a regular latticework on the dorsal surface and a more irregular network ventrally.

2. These lines are associated with rows of rosettes from which bristles protrude on both dorsal and ventral surfaces. These bristles are thought to be sensory in function and the network a sensory conductor system.

3. The neuromotor system was studied and Yocum's description supported except for the motorium. Evidence is presented which seems to indicate that there is no motorium in this species.

4. The external network is directly connected with the neuromotor system.

5. Discussion brings out the probability that the neuromotor apparatus in *E. patella* is thus augmented by a distinct but connected external network of sensory fibrils.

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SELECTIVE ACCUMULATION OF IONS IN CAVITIES INCOMPLETELY SURROUNDED BY PROTOPLASM

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Selective accumulation of ions in tissue fluids has usually been thought of as a result of more or less complete enclosure of the fluid within a continuous layer of protoplasm or plasma membrane. Accordingly, when a body fluid containing principally a few selected ions is separated from the external medium by an epithelium, the cells of this epithelium are supposed to press so closely one against the other that ions can only cross through the epithelium by traversing plasma membranes and cytoplasm. In like manner, the Casparian strips have been thought to block essentially all intercellular paths between the soil solution and the sap in the stele of the vascular plant. A study of the fluids of the marine green alga *Codium Bursa* (L.) Ag. (Order: Siphonales) shows that assumptions of this type are not necessary, since in the case of *Codium* there is selective accumulation of ions in the fluid of the central cavity, which is open to the sea water by intercellular paths.

A brief description of the plant may be given here. Its thallus is in the form of a hollow oblate spheroid a few centimeters in diameter, attached by rhizoids at the base. The walls of the spheroid are a few millimeters thick, and consist of two layers. The internal layer is made up of intertangled basal filaments running for the most part in a tangential direction. From these there arises as an external layer a dense felt of clavate radial filaments aptly referred to as "palisade cells." The walls of these cells are made up of pectins with some cellulose, and are considerably thickened at the distal ends. Thickenings on the interior of the cell walls obstruct free movement of material at various points, notably in the basal filaments close to the branching of each palisade cell. A strand of protoplasm is supposed to penetrate through these thickenings, but Arcangeli reports that pressure exerted on the filaments to one side of a thickening could not be made to force material past it. (Arcangeli, 1874.) In any event a vacuole extends continuously from near the tip of each palisade cell through at least some portion of a basal filament. Turgor of the palisade cells is marked, and although not mutually adherent, they are closely pressed together.[1] Published descrip-

[1] The description is taken for the most part from F. Oltmann's *Morphologie und Biologie der Algen*, II Aufl., Jena, 1922, where further details may be found.

tions make no mention of canals opening to the exterior from the central cavity, although such things are said to be present in related species. To make sure about this, specimens of *Codium Bursa* were wiped dry with filter paper and then squeezed. No fluid was forced out of the plant in this way. The central cavity of *C. Bursa* has therefore no obvious direct connection with the sea water through orifices or canals.

This makes it appear that ions can pass into the central cavity from the surrounding sea water by either of two paths, traversing, respectively, the protoplasm and the cell wall material. Whatever the path by which ions enter the central cavity, it is clear from Table I that a selective process occurs at some point, since K is present in the central cavity

TABLE I

The concentrations of K, Na, and Cl in the cell sap, and in fluid from the central cavity of *Codium Bursa* (L.) Ag., and in the surrounding sea water.

Fluid	Concentration, millimols per liter			Ratio of K to Na
	K	Na	Halide	
Cell sap	84	627	657	0.134
Central cavity	61	567	624	0.108
Central cavity	64	576	619	0.111
Sea water *	22	641	656	0.034

* The sea water was taken from the aquarium system in which the plants were immersed.

in higher concentration than in the surrounding sea water, while the Na concentration is lower. The halide concentration is slightly higher, presumably because SO_4, which was not separately determined, is partially excluded.[2]

Table I also shows that sap exuded under pressure from cut edges of thallus is still more modified in the direction of selective accumulation of K than is the fluid in the central cavity. The sample of sap is presumably mixed with a good deal of sea water and other fluids, and the pure cell sap probably contains K in even higher proportion than indicated in the table.

It therefore appears probable that the selective process results during the passage of ions from the sea water into the cells, and perhaps also on passage from the cells into the fluid of the central cavity. Restricted but non-selective interchange of ions along the cell walls would

[2] Halide was determined by titration with $AgNO_3$, Na + K as total sulfate, K as chlorplatinate, and Na by difference.

account for the fact that the fluid of the central cavity is more like sea water than is the cell sap.

It must be concluded, therefore, that some accumulation of ions may occur in fluids which are only partially separated from the surrounding medium by living protoplasm. Such tissues as the endodermis of roots of vascular plants, the gill epithelia of fishes, or the epithelium of the convoluted tubules of the mammalian kidney may fail to form a continuous wall of protoplasm, and still give rise to what is known as the selective accumulation process, viz., the partial exclusion of some ions, and the movement of other ions through the tissue against their own activity gradients.

The plants for these analyses were obtained at the Stazione Zoologica at Naples, where the writer occupied the Woods Hole-Columbia University table. The analytical work was supported by a grant from the National Research Council. Grateful acknowledgment is made for these aids.

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REGENERATION IN THE CARAPACE OF DAPHNIA MAGNA

I. The Relation between the Amount of Regeneration and the Area of the Wound during Single Adult Instars

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In all animals the rates of regeneration appear to have identical characteristics. Przibram (1919) cited the experiments of eleven workers on twenty species and reported that the rates of regeneration are most rapid at the beginning of the process and decrease as regeneration continues. After a study of twelve diverse species, Zeleny (1909*a*) arrived at the conclusion that the rates of regeneration vary directly with the degree of injury up to a maximum, beyond which the rates decrease. Carrel and his colleagues (1916, etc.) found that wound closure in man follows the same general trend, in that the rate of cicatrization is proportional to the area of the wound, but diminishes less rapidly than the area.

The above-mentioned works deal with regeneration of complex structures composed of many tissues and consequently treat with a composite of the rates of individual tissues. A study of the rate in a less complex structure should lead to a better understanding of the fundamental problems involved.

The carapace of *Daphnia magna* is a comparatively simple structure. Two layers of hypodermis (Fig. 1) constitute the cellular tissue of the ventral half of the carapace posterior to the shell gland, the region which is injured by the operative procedure outlined below. Between these layers is a blood chamber and associated with them on their outer surfaces is the chitinous integument. The pillars, which probably also consist of chitin, serve as supporting structures. Figure 2 is a surface view showing the arrangement of the outer hypodermal cells. The markings of the carapace which are characteristic of this cladoceran conform closely to the cell boundaries. The pillars do not appear to be arranged in any definite order.

Sections of the regenerated portions of the carapace are identical with those of the uninjured regions. When viewed from the surface, the shape of the regenerated hypodermal cells is quite variable, in

[1] The greater part of the experimental work upon which this paper is based was carried out at the Zoölogical Laboratory of the State University of Iowa.

contradistinction to that of the original cells. Since the carapace remains the same in cross-section, the difference in the area of the wound from one time to another serves as a three-dimensional measure of the amount of regeneration during the interval.

The purpose of this paper is to present a study of the amount of regeneration in the carapace during single physiological time units, *i.e.* the instars, in relation to the size of the wound under varying conditions

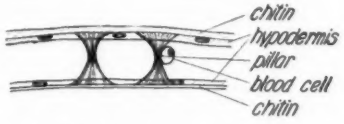

FIG. 1. Camera lucida drawing of a cross-section through the carapace.

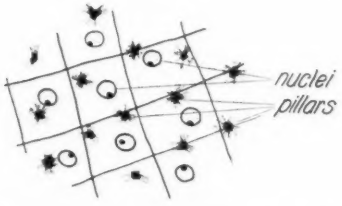

FIG. 2. Camera lucida drawing of the outer surface of the carapace showing the typical arrangement of cells. The surface markings correspond to the cell boundaries. The pillars appear in no definite order.

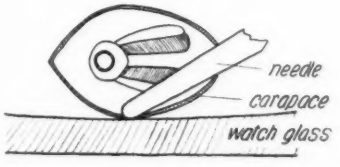

FIG. 3. Diagrammatic section through an animal showing method of operation. Injury to structures other than the carapace is avoided.

of age of the animal, age of the wound, and temperature. These results will be discussed in relation to those secured by others for more complex structures in other forms.

The writer wishes to acknowledge his indebtedness to Dr. L. A. Brown and Dr. J. H. Bodine—to the former for suggesting the problem, and to both for their many helpful suggestions and criticisms as the work progressed.

Materials and Methods

The material, *Daphnia magna* Straus., was secured from Banta's laboratory in 1927. Females from three clones were employed. The original clone was used in the experiments on the effect of age but died out late in 1928. A new clone, started with ephippial eggs from the former in January, 1929, was used in the experiments dealing with temperature effect. Another clone, derived from the latter, was used in the experiments concerned with age of the wound.

Individuals were isolated within twenty-four hours after their re-

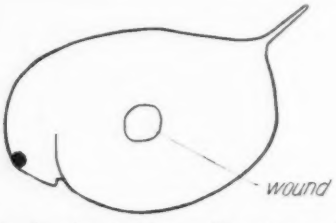

Fig. 4. Outline drawing showing position of wound in carapace.

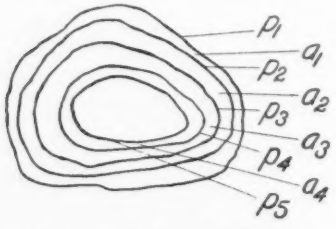

Fig. 5. Camera lucida drawings of a wound during five successive instars. p_1 represents the edge of the wound during the first post-operative instar; p_2, the second; etc. The area a_1 represents the amount of regeneration during the first post-operative instar; a_2, the second, etc. In the graphs which follow, the amount of regeneration during each instar is plotted against the area of the wound during that instar, *i.e.*, a_1 against total area enclosed by p_1; etc.

lease from the brood chamber of the mother and placed in vials containing fresh manure-soil medium (Banta, 1921). The vials used in the experiments on the effect of age and of temperature contained thirty to thirty-five cc. of medium, while those used in the experiments on the effect of the age of the wound contained fifty to sixty cc. of medium. Semi-weekly throughout the experiments one-third of the fluid in each vial was removed and replaced by fresh culture medium.

In the experiments to determine the effect of the temperatures 15° C. and 25° C. the animals were placed, immediately after isolation from

the mother, in an electrical refrigerator fitted with a mercury thermoregulator, a heating element, and an electric fan (air temperature ± 0.5° C.). All other experiments were carried on at room temperature (18°–25° C.).

In the operative procedure the animals were placed in watch-glasses and immobilized with a chloretone solution of a concentration just sufficient to bring about cessation of movement. The chloretone did not

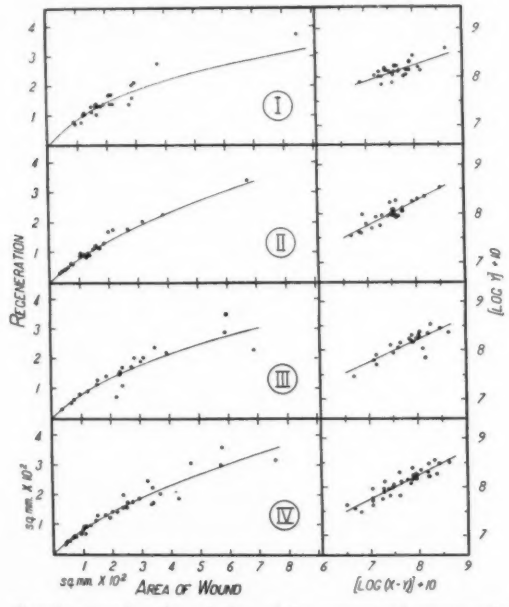

FIG. 6. Graphs showing the relation of the amount of regeneration to the area of the wound during adult instars I–IV. Each animal was operated early in the instar before the one in question. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

For comparison with later adult instars see Fig. 8.

appear to have any detrimental effects. Part of the area of the carapace in contact with the watch-glass was crushed by applying a steel needle to its inner surface (Fig. 3). Figure 4 shows the position of the wound. The proportions of the wounds were varied by using needle points of different sizes and shapes. Approximately 4000 individuals were operated upon in these experiments.

During the early part of each instar following operation the animals were again immobilized with chloretone and camera lucida drawings (175 ×) made of the wounds. From these drawings the areas were determined by the use of compensating polar planimeters (Keuffel & Esser Co., No. 4242 and No. 4240) and the perimeters by a measuring wheel (Keuffel & Esser Co., No. 1694A) graduated to 1/32 inch.

Experimental

A few hours after an injury is inflicted on the carapace of *Daphnia magna* a brown material forms at the edge of the damaged area. In

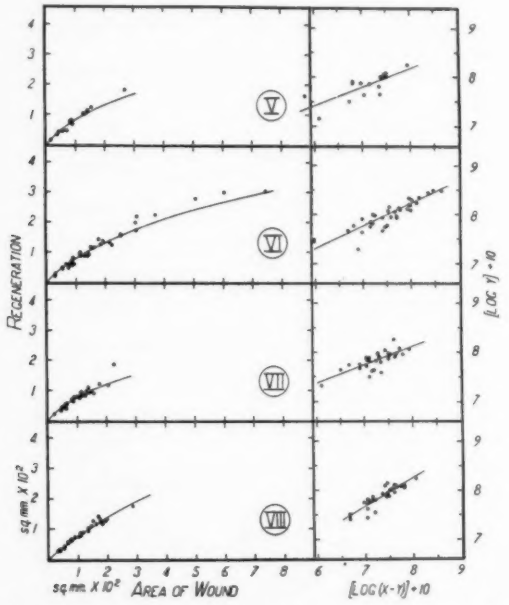

Fig. 7. Graphs showing the relation of the amount of regeneration to the area of the wound during adult instars V–VIII. Each animal was operated early in the instar before the one in question. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

For comparison with other adult instars see Fig. 8.

all probability this brown material is clotted blood in which the tyrosine has been oxidized (Pinhey, 1930; Anderson, 1932*b*). The brown ma-

terial is shed with the chitin at the next ecdysis when the wound is made before three-fourths of the instar has passed. However, if the injury is made after three-fourths of the instar has passed, the brown material is retained until the second molt following the injury. If an injury is inflicted early in the instar the edge of the wound during the next instar is smooth and clear. If the injury occurs after half but before

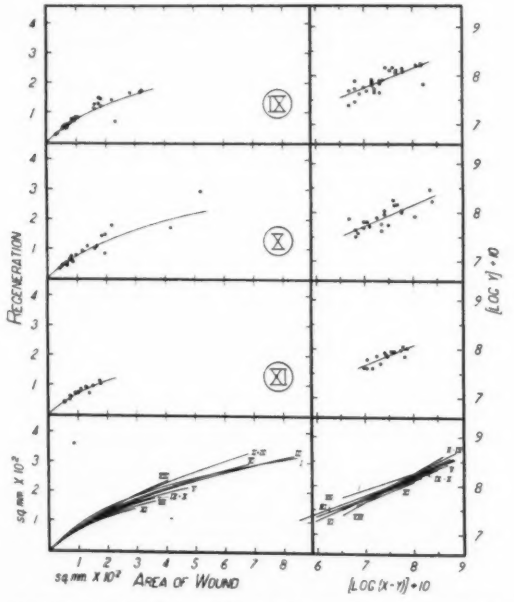

FIG. 8. Graphs showing the relation of the amount of regeneration to the area of the wound during adult instars IX–XI. Each animal was operated early in the instar before the instar in question. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

The bottom graphs give the curves for each of the adult instars I–XI for purposes of comparison.

three-fourths of the instar has passed, the edge of the wound is rough during the following instar. These phenomena appear to be directly related to the presence or absence of a second layer of chitin at the time of injury (Anderson and Brown, 1930). In all cases, no matter what be the nature of the wound during the first post-operative instar,

the edge of the wound is smooth and clear throughout the remaining instars, *i.e.* until complete closure results.

Further, when an operation is performed within the first half of the instar some regeneration takes place before the animal molts. The area of the wound during the following instar is less than that included within the brown ring during the instar of operation. If an operation is performed within the period after half the instar has passed and before three-fourths is over, the wound during the next instar is approximately the same size as the area enclosed by the brown material during the operative instar. In case the injury is produced after three-fourths of the instar has passed, the brown material occupies the same area during the next instar as it does during the instar of injury. Normally wounds diminish noticeably in size by the second post-operative instar irrespective of the time within the instar of operation that the injury is inflicted.

TABLE I

Values of the constants in the equation $y = a(x - y)^k$ expressing the relation between the amount of regeneration (y) and the area of the wound (x) for first post-operative instars with reference to the age of the animals. The data on which these constants are based are shown in Figs. 6, 7, and 8.

Adult instar	Constants		Number of cases
	a	k	
I	.09	.35	29
II	.18	.50	29
III	.13	.46	23
IV	.18	.50	35
V	.09	.38	16
VI	.12	.45	34
VII	.07	.37	31
VIII	.27	.59	30
IX	.09	.40	30
X	.10	.43	23
XI	.08	.42	15

The wound remains the same in size and shape throughout any one instar. Change takes place only at ecdysis and shortly thereafter. This is also true for the body length and for the antennal segments of the animal (Anderson, 1930, 1932*a*, *b*) and for the antennæ of *Simocephalus gibbosus* and *Daphnia carinata* (Agar, 1930).

Preliminary experiments showed the impracticability of securing wounds of an exactly identical size and shape. The area injured was always greater than the immediate area crushed. This extended area

of injury varied considerably for each case. These experiments also showed that the amount of regeneration during any instar varied with the size and shape of the wound. The shapes may be classified according to the ratios of the perimeter to the square root of the area. The data included in this paper are for wounds where the ratio of the perimeter to the square root of the area does not exceed four. The wounds coming under this classification range from approximate circles to elongated ovals whose lengths rarely exceed twice their widths.

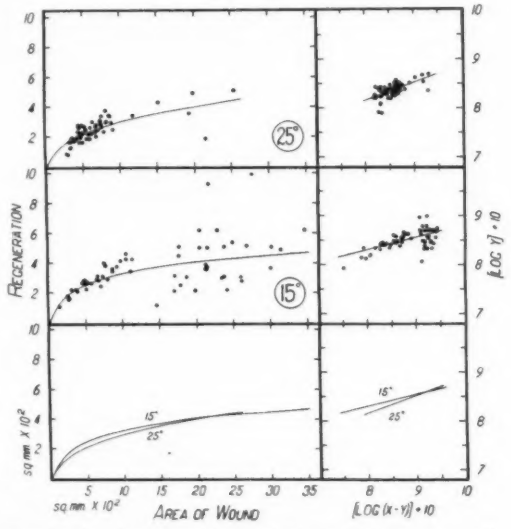

FIG. 9. Graphs showing the relation of the amount of regeneration to the area of the wound during adult instar II at the temperatures 15° and 25° C. Each animal was operated early in the instar before the one in question. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

With the foregoing facts in mind, a series of experiments was designed to determine the amount of regeneration for wounds of different sizes but of the same approximate shape, controlled by the means outlined above, during each of the first eleven adult instars. In each case the animals were operated early in the instar previous to the one for which records were taken. The results are shown in Figs. 6, 7, and 8. The area of the wound represents size during the instar in

TABLE II

Values of the constants in the equation $y = a(x - y)^k$ expressing the relation between the amount of regeneration (y) and the area of the wound (x) for first post-operative instars with reference to temperature, the age of the animals being the same. The data on which these constants are based are shown in Fig. 9.

Temperature	Constants		Number of cases
	a	k	
°C.			
15	.06	.25	62
25	.08	.36	56

question. The amount of regeneration is the difference between the area of the wound during the instar in question and the area during the following instar (Fig. 5). The results indicate that the amount of regeneration for wounds of identical sizes is approximately the same during the adult instars studied.

Mere graphic presentation of the data yields only a rough means of comparing the results. It was therefore deemed advisable to determine the nature of the relation between the amount of regeneration and the area of the wound. On first glance the relation seems parabolic. The simplest expression for a parabolic curve would be

$$x = ay^k. \tag{1}$$

But the amount of regeneration can never exceed the area of the wound. By adding y to the second member of equation (1) we have

$$x = ay^k + y. \tag{2}$$

By letting y be the amount of regeneration and x be the area, the amount of regeneration can never exceed the area of the wound.

For ease of manipulation, since both x and y are known, equation (2) was converted to

$$y = a(x - y)^k. \tag{3}$$

If this equation is suitable, double logarithmic plots of the amount of regeneration (y) against the area minus the amount of regeneration ($x - y$) should yield a straight line.[2] Such is practically the case as is shown in Figs. 6–11.

At present this equation may be considered only descriptive. No biological significance has been assigned to the constants.

[2] It may be noted that $(x - y)$ is the area of the wound after y has been regenerated.

The constants for each experiment were determined by the method of least squares. The curves in the figures were fitted by means of the constants so determined.

A study of the composite graphs in Fig. 8 and the constants in Table I shows that in general for wounds of the same size the amount of regeneration decreases as the animals grow older.

The amount of regeneration for wounds of identical sizes and shapes is less during the pre-adult instars than during adult instars. The amount of regeneration increases with each instar until the adult condition is reached. The first adult instar is the one during which

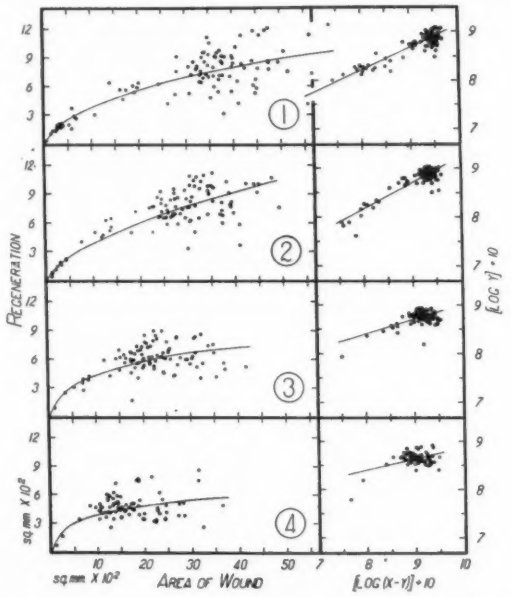

FIG. 10. Graphs showing the relation of the amount of regeneration to the area of the wound for each of the first four instars after operation. Each animal was operated early in the first adult instar. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

For comparison with later instars see Fig. 11.

the first clutch of eggs appears in the brood chamber. Considerable irregularity has been found in the number of pre-adult instars for this

species (Anderson, 1930, 1932*a*). Consequently comparisons of the amounts of regeneration for any given pre-adult instar are hardly justifiable at present.

Another series of experiments was carried out to test the effect of temperature on the amount of regeneration during a single instar. Animals which had been kept at specified temperatures (15° and 25°

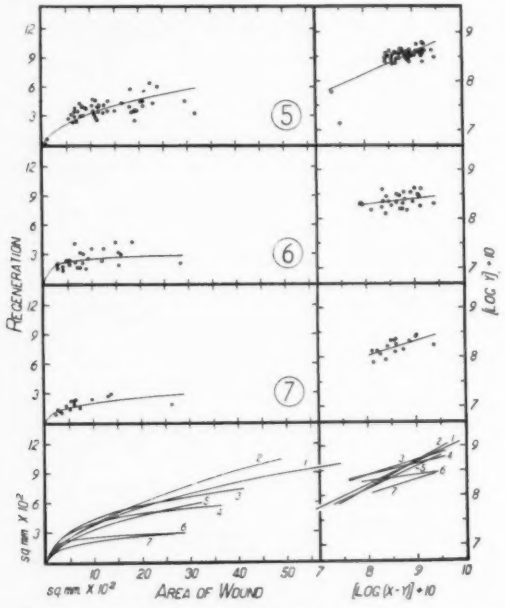

FIG. 11. Graphs showing the relation of the amount of regeneration to the area of the wound for the fifth to the seventh instars after operation. Each animal was operated early in the first adult instar. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

The bottom graphs give the curves for each of the first seven instars following operation.

C.) from the time of their isolation were operated during the early part of the first adult instar. Records were taken for the second adult instar. The results are shown in Fig. 9. The constants are given in Table II. From the figure it may be seen that the difference in temperature within the limits studied has relatively no effect on the amount of regeneration.

The preceding experiments were concerned with relatively small wounds and with the first post-operative instar. Another series of experiments was designed to determine the amount of regeneration for the largest possible wounds and further to determine the effect of the age of the wound on the amount of regeneration. Animals were operated early in the first adult instar and records taken during each successive instar until death ensued. The results are shown in Figs. 10 and 11. The size of the wound is always that during the instar in question as shown in Fig. 5. The results for the eighth and ninth post-operative instars are not shown, since the number of cases was considered too small to be of significance. The values of the constants for each post-operative instar are given in Table III.

TABLE III

Values of the constants in the equation $y = a(x - y)^k$ expressing the relation between the amount of regeneration (y) and the area of the wound (x) for successive instars following operation. The data on which these constants are based are shown in Figs. 10 and 11.

Post-operative instar	Adult instar	Constants		Number of cases
		a	k	
1	II	.14	.45	105
2	III	.17	.54	102
3	IV	.10	.30	94
4	V	.08	.23	79
5	VI	.11	.45	51
6	VII	.03	.12	28
7	VIII	.04	.30	15
8	IX	.03	.25	10
9	X	.06	.47	5

The results show that the relation tends to be the same for large and for small wounds, during the first post-operative instar. The values of the constants for the first few post-operative instars are almost the same as those where the age of the animal alone was considered. A study of the composite graph in Fig. 11 shows that the amount of regeneration decreases with the increase in the number of instars between the time of operation and the instar during which records were taken and to a greater extent than that where age of the animal alone is considered.

DISCUSSION

Zeleny (1909*a*) pointed out the principal sources of error that arise in quantitative work on regeneration. As he listed them they are: (1)

age, (2) periodic physiological changes, (3) character of the laboratory histories, (4) changes in the rate during the regeneration period, (5) successive regenerations, (6) level of the cut, (7) temperature, (8) food, (9) differences in manipulation, (10) departure of living conditions from the optimum, (11) the relation of the degree of injury to the optimum degree, (12) individual variation. Five of these factors have been made subjects of the present study, namely: age, periodic physiological changes—the instar, changes in rate during the regeneration period, temperature, and relation of the degree of injury to the optimum degree. The other factors have been eliminated in the following manner:

The Character of the Laboratory History.—The animals are well adapted to laboratory conditions since they have been reared in laboratories for well over a decade.

The Level of the Cut.—Zeleny had reference to the amount removed. This factor may be thought of in a somewhat different sense in the present case, for the various regions of the carapace may have different rates of regeneration. The positions of the wounds were as nearly identical as possible in all instances.

Successive Regeneration.—The problem at hand is not concerned with this factor.

Food.—The food used was as uniform as possible. Banta's manure-soil medium was employed in all experiments.

Differences in Manipulation.—As far as possible all individuals in any one series of experiments were subjected to the same manipulations. (See *materials and methods.*)

Departure of Living Conditions from the Optimum.—All animals in any one series of experiments were subjected to the same living conditions. The animals retained their vigor as was evidenced by the continued production of young throughout the experiments.

Individual Variation.—This factor was reduced to a minimum through the use of animals from a single clone in each series of experiments, thus insuring individuals genotypically alike. Large numbers of animals were employed as may be seen from the tables.

Zeleny in his treatment of the above factors suggests that the molting period be used as the center of observations for Crustacea. Indeed, such is the only possible way to handle the above problem. The size and shape of the animals remain constant throughout any one instar. Only at ecdysis and within the period immediately following, during which the chitin hardens, does any normal change take place. The wound remains the same in size as does the carapace of the animal.

Whether or not adult instars are quantitatively equivalent units is a question which remains unsolved. Under optimum conditions females bear a brood of young during each adult instar. At the beginning of each adult instar a number of eggs are passed into the brood chamber. These develop into free-swimming young which are released a few hours before the end of the instar. Usually the time between the release of the young and the end of the instar increases with the age of the animal. The duration of an instar also probably increases with age.

MacArthur and Baillie (1929) and Obreshkove (1930), working with *Daphnia magna* and *Simocephalus expinosus* respectively, found that the metabolic rate decreases with age. If adult instars are quantitatively equivalent units one would expect that the instars would be proportionately longer as the metabolic rate decreases. However, since the amount of regeneration is somewhat less for wounds of the same size during later adult instars than for earlier, one might come to the conclusion that instars are not necessarily equal. Further work on instar length in relation to metabolic rate is necessary to determine the status of the adult instars as quantitatively equivalent units.

Zeleny (1909*a*) found that the rate of regeneration of an organ increases with the degree of injury up to an optimum beyond which the rate decreases. In his experiments several organs were removed and the rate of regeneration for one was compared to the rate when that one only was removed. One must note in the present results that the amount of regeneration for any instar increases with the size of the wound, but the ratio of the amount of regeneration to the area of the wound decreases as the area is increased.

Zeleny (1909*b*) also noted the rate of regeneration for younger animals to be less than for older, but the actual time necessary to replace an organ to be more for older than for younger animals. Du Noüy (1916) showed that the rate of cicatrization in man decreased with the age of the individual. In the case at hand the amount of regeneration during the first post-operative instar for wounds of equal size in adult animals is more in younger than in older animals.

Przibram (1926) has regarded regeneration as an acceleration of normal growth processes. This viewpoint is tenable if the rate of regeneration need not be dependent on the rate of normal growth. Increase in size of *Daphnia magna* females is greatest during the first two adult instars and becomes less with each adult instar up to the tenth, after which time the size of the animals remains relatively constant (L. A. Brown, unpublished data). It may be observed in Fig. 8 that the amount of regeneration decreases with age. The decrease in the amount of regeneration is not equivalent to the decrease in the

amount of growth. Godlewski and Latinik (1930) reported that in the tail of the axolotl growth and regeneration are largely independent of each other.

Durbin (1909) found that the rate of regeneration in tadpole tails was quite slow at first but increased to a maximum and decreased to zero after a time. Przibram (1919) concluded from experiments on twenty species from five different phyla that the rates of regeneration are most rapid at the beginning of the process and decrease as regeneration continues. A study of Figs. 10 and 11 shows that the amount of regeneration is greatest at first and decreases as the process continues.

The results here presented agree in general with the findings of Carrel and Hartmann (1916) for the cicatrization of wounds in man, in that the rate is proportional to the area of the wound.

Ebeling (1922) reported that the value of the Q_{10} was 2 for the rate of cicatrization of wounds in alligators. The value of the Q_{10} for the instar length of young adult female *Daphnia magna* is approximately 2.3 between the temperatures 15° and 25° C. (Anderson and Brown, 1930). While instar length varies directly with the temperature, the amount of regeneration is relatively unaffected. The rate of regeneration taken on the basis of absolute time units would therefore be affected by temperature to the same degree as is instar length.

One may conclude that the amount of regeneration during any single adult instar is a parabolic function of the area of the wound during that instar, and is independent of the temperature, but decreases with the age of the animal and to an even greater extent with the age of the wound.

Summary

1. This report deals with a study of the amount of regeneration in the carapace of *Daphnia magna*.

2. The amount of regeneration during any adult instar is a parabolic function of the area of the wound during that instar and may be expressed by the equation

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants.

3. The amount of regeneration during any adult instar decreases with the age of the animal.

4. The amount of regeneration during adult instars is independent of the temperature.

5. The amount of regeneration during any adult instar decreases with the age of the wound but to a greater extent than when the age of the animal alone is considered.

6. These results have been discussed with reference to those secured by others for relatively complex structures.

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MITOSIS IN HYDRA

Mitosis in the Ectodermal Epithelio-Muscular Cells of Hydra

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Introduction and Historical

Various workers with *Hydra* have come to the conclusion that the ecto-epithelio-muscular cells of *Hydra* do not divide, and that if mitotic figures are found in them it is the exception rather than the rule. Downing (1905) says, "In all the sections studied mitosis has been the universal mode of division in the interstitial cells, the exceptional mode in the ectoderm cells and amitosis the constant rule in the endoderm cells. Fig. 5 shows an ectoderm cell in process of amitotic division. At first it was thought this was the only mode of division of the ectoderm cells. But a few cases have been observed, only two or three in hundreds of sections, however, where mitosis occurred in an undoubted ectoderm cell. Schneider had observed and figured mitosis in an ectoderm cell. But cell multiplication, except of the interstitials seems to be almost invariably amitotic." (Pages 411–412.)

Strelin (1928) says, "K. C. SCHNEIDER (1890) findet in seiner eingehenden zytologischen Untersuchung gar keine Übergangszellenformen zwischen den I. Z. einerseits und den Epithelmuskelzellen anderseits; deshalb ist er geneigt zu denken, das die Neubildung der Epithelmuskelzellen auf Kosten der Teilung der differenzierten Elemente stattfindet. Als Bestätigung führt SCHNEIDER die Teilungsbilder der Epithelmuskelzellen an.

"Vielen Autoren ist es aber gar nicht gelungen, in den spezialisierten Zellen der Hydra Mitosen zu beobachten; ich konnte ebenfalls in diesen Zellen kein einziges Mal Mitosen beobachten, obgleich ich zu diesem Zwecke eine grosse Zahl von Schnitten durchgesehen habe. SCHNEIDER (1890), STECHE (1911), GELEI (1924), MATTES (1925), KANAJEIO (1926) u.a. haben in den zpezialisierten Zellen Mitosen angetroffen, sie halten aber dieselben für eine seltene Erscheinung. Es ist daher kaum möglich, die Neubildung der Epithelzellen ausschliesslich durch die Vermehrung derselben zu erklären." (Page 274.)

We see then that Strelin maintains that the indifferent cells are transformed into ecto-epithelio-muscular cells in mitosis. He further

states, " Die Frage darüber, auf welche Weise aus einer ganzen Gruppe kleiner junger Epithelzellen nur mehrere Elemente entstehen, ist für mich nicht ganz klar, um so mehr, als die Zahl der Zellen sich stets durch Vermehrung vergrössern muss. Es ist möglich, dass im gegebenen Falle die Rückentwicklung der jungen Epithelzellen in Interstitiellzellen stattfindet; es ist aber viel wahrscheinlicher, dass einige junge Epithelzellen auf den Anfangsstadien ihrer Entwicklung aufgehalten werden. In den grösseren von den jungen Epithelzellen kommen ebenfalls, wie oben vermerkt wurde, Mitosen vor; man muss aber bemerken, dass je mehr die Zellen differenziert sind, desto seltener in ihnen Mitosen vorkommen; in den erwachsenen Epithelzellen ist es mir niemals gelungen, wie ich bereits erwähnt habe, Mitosen zu begegnen." (Pages 278–279.) We see further, that Strelin noticed the difference in the size of the indifferent cells, but he calls the larger of these, which he found in mitosis, " jungen Epithelzellen " and thinks that when these " jungen Epithelzellen " are further transformed into ecto-epithelio-muscular cells they never divide.

McConnell (1930 and 1932) has demonstrated the mitosis of the endo-epithelio-muscular cells and made reference to the mitosis of the ecto-epithelio-muscular cells. Owing to the fact that so many mitotic figures have been found (and easily found) in the ecto-epithelio-muscular cells, it was decided to make a study of this process to clarify the question of mitosis in these cells.

Materials and Methods

Pelmatohydra oligactis was used in this study. The material was collected in the vicinity of the University of Virginia, Charlottesville, Virginia, U. S. A.; the University of Ljubljana, Ljubljana, Jugoslavia; the Kaiser Wilhelm-Institute for Biology, Berlin-Dahlem, Germany. Preparations studied were as follows: without buds 105, with buds 75, with testes 15, with ovaries and testes 7, with ovaries 3. These *Hydra* were killed and fixed in the following solutions: in Bouin's, in Flemming's, in Carnoy's acetic alcohol, and in Zenker's. Sections were cut at five and seven microns. Staining was done on the slide with iron-hematoxylin and acid fuchsin, with Delafield's hematoxylin, and Bordeaux red; methyl violet and water-soluble eosin were also used in some instances. The preparations were mounted in Damar and Canada Balsam. They were studied with a compound binocular microscope equipped with 430 × dry and 97 × oil immersion objectives; 5 ×, 10 ×, and 15 × paired oculars were used. Microphotographs were made at magnifications of 430 ×, 860 ×, and 970 ×, during which processes an Orsam point-light was used.

Prophase

During the prophase the nucleus enlarges and moves slightly toward a central distal position; it takes nuclear stains much more intensely than do the normal resting nuclei. (See Fig. 3, Plate I.) The number of nucleoli varies from one to four, and they remain evident up until the beaded spireme is formed. The entire cell rounds up and the muscular processes are withdrawn from the mesoglea. This rounding-up process gives a characteristic swollen appearance to the area where the mitosis is taking place. The beaded spireme is very active in the nuclear area and may be seen, in maceration preparations, to be very actively whirling about. There are twelve chromosomes of V-shape.

Metaphase

The chromosomes line up on the equatorial plate with the tips of the V's pointed to the center. The asters and centrioles are quite prominent. The chromosomes start dividing at the tip of the V, and continue dividing longitudinally. As the open arms of the daughter chromosomes recede, they remain connected by spindle fibers.

Anaphase

As the daughter chromosomes approach the division centers the tips of the V's fuse slightly, with the ends of the daughter chromosomes protruding in various directions. As they move towards the pole the centriole becomes more and more indistinct and gradually disappears.

Plate I

1. Resting nucleus with four nucleoli. Bouin's; iron-hematoxylin. 776 ×.
2. Early prophase to the left, and daughter cell to the right. Bouin's; iron-hematoxylin, and acid fuchsin. 776 ×.
3. Lower left, normal resting nucleus; upper right, later stage of prophase. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.
4. Prophase just prior to the disappearance of nuclear membrane. Bouin's; iron-hematoxylin. 776 ×.
5. Same as No. 4. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.
6. The metaphase chromosomes. Bouin's; iron-hematoxylin. 776 ×.
7 and 8. Two metaphase stages. Notice the slight bulge which makes this stage very noticeable. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.
9. Metaphase from the base of tentacle. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.
10. Metaphase. Fleming's; iron-hematoxylin and acid fuchsin. 776 ×.
11. Metaphase. Carnoy's acetic alcohol; iron-hematoxylin and acid fuchsin. 776 ×.
12. Metaphase perpendicular to mesoglea. Zenker's; iron-hematoxylin and acid fuchsin. 776 ×.

PLATE I

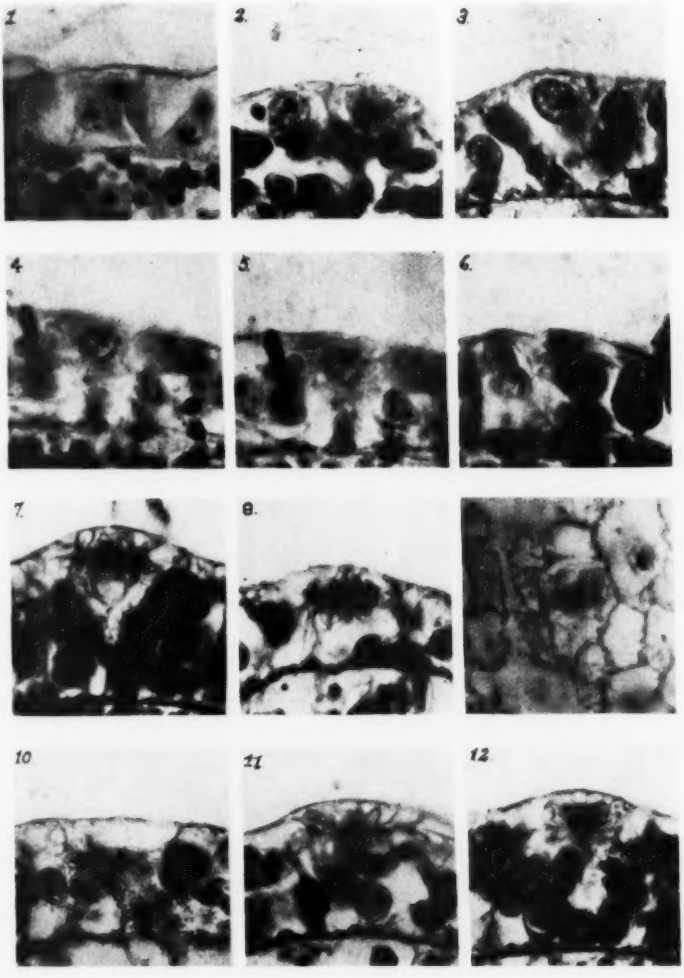
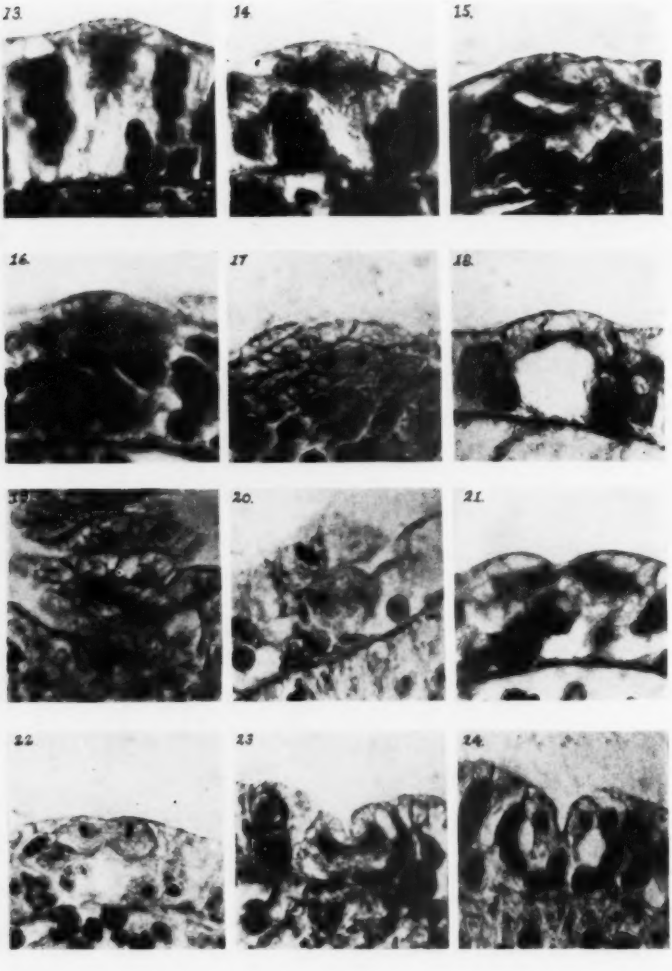


PLATE II



Telophase

Upon reaching the area where daughter nuclei will be formed the chromosomes expand very quickly and reconstruct nuclei. The cleavage furrow is most noticeable in mitotic figures in areas where the cells are not closely crowded together or when the animal has been killed and fixed in an expanded condition. After the daughter cells are separated and the nuclei are in the process of being reconstructed,

Table I

	No. of preparations	Prophase		Metaphase		Anaphase		Telophase	
		early	late	early	late	early	late	early	late
With ovaries and testes	7	12	13	11	10	2	4	9	6
With ovaries	3	4	9	17	12	6	2	14	27
With testes	15	42	17	31	19	4	9	20	14
With buds	75	92	27	46	24	4	17	29	34
Without buds	105	101	47	30	21	7	27	49	10
Total	205	251	113	135	86	23	59	121	91

that part of the cell which lies nearest the mesoglea begins to push its way down between the surrounding cells toward the mesoglea; and, when this is reached, forms muscular processes.

Occurrence of Mitosis

As stated above, two hundred and five preparations were studied; Table I, showing the different stages, would seem to indicate that mitosis is the rule rather than the exception. Thus we find a total of 879 mitotic figures in 205 preparations.

Plate II

13. Metaphase perpendicular to mesoglea. (Compare with No. 7, Plate I.) Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.
14. Anaphase. Fleming's; Delafield's hematoxylin. 776 ×.
15. Anaphase. Carnoy's acetic alcohol; iron-hematoxylin and acid fuchsin. 776 ×.
16. Anaphase. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.
17. Anaphase. Fleming's; iron-hematoxylin. 688 ×.
18. Anaphase. Note large vacuole between achromatic figure and mesoglea. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.
19. Anaphase from base of tentacle. Bouin's; Delafield's hematoxylin. 776 ×.
20. Anaphase. Bouin's; iron-hematoxylin. 688 ×.
21. Telophase. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.
22. Late anaphase. Fleming's; iron-hematoxylin. 688 ×.
23. Telophase. Zenker's; Delafield's hematoxylin. 776 ×.
24. Telophase from tip of growing bud. Fleming's; iron-hematoxylin and acid fuchsin. 776 ×.

These mitotic figures are found in all parts of the *Hydra's* body except in the epithelio-muscular cells of the tentacles. The number of mitotic figures varies greatly as to the region of the body; the majority are found in all cases in the upper two-thirds of the body; however, many may be found in the so-called "stalk-region" and on the peristome. These dividing cells may also be found in the basal disc, but this appears to be rather rare.

In the majority of cases, the achromatic figure during the metaphase is parallel to the mesoglea; however, in some exceptional cases, one finds the metaphase spindle arranged perpendicularly to the mesoglea, in which case one of the daughter cells is buried under the surrounding epithelial cells; the fate of these buried cells has, as yet, not been determined. (Fig. 12, Plate I, Fig. 13, Plate II, and Fig. 36, Plate III.)

Discussion

Kanajew (1930), who worked quite extensively on the problem of regeneration in *Hydra*, and who reviewed all of the literature on regeneration, says: "Mitosen sind nur in den I-Zellen häufig. In differenzierten Ekto- und Endodermzellen habe nur seltene Falle der Zellvermehrung bemerkt." Since Kanajew was working on regenerating *Hydra* and not *Hydra* in their normal condition, it is quite natural that he should not find many cases of the mitosis of the ecto- and endo-epithelio-muscular cells. The indifferent interstitial cells, as he noted, are found in mitosis in *Hydra* under all conditions of experimentation.

Threlkeld and Hall (1928), in their experiments upon *Hydra* under determined ion concentration, starved *Hydra* over long periods of time.

Plate III

25. Telophase. Zenker's; iron-hematoxylin. 776 ×.

26. Telophase. Carnoy's acetic alcohol; Delafield's hematoxylin. 776 ×.

27. Telophase from tip of bud. Note remains of spindle fibers. Bouin's; iron-hematoxylin. 688 ×.

28. Telophase. Note remains of spindle fibers. Bouin's; Delafield's hematoxylin. 688 ×.

29. Late telophase, from peristomal region. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.

30, 31, and 32. Stages at which the daughter cells begin growing down toward the mesoglea, where muscular processes will be formed. Bouin's; Delafield's hematoxylin and Bordeaux red. 776 ×.

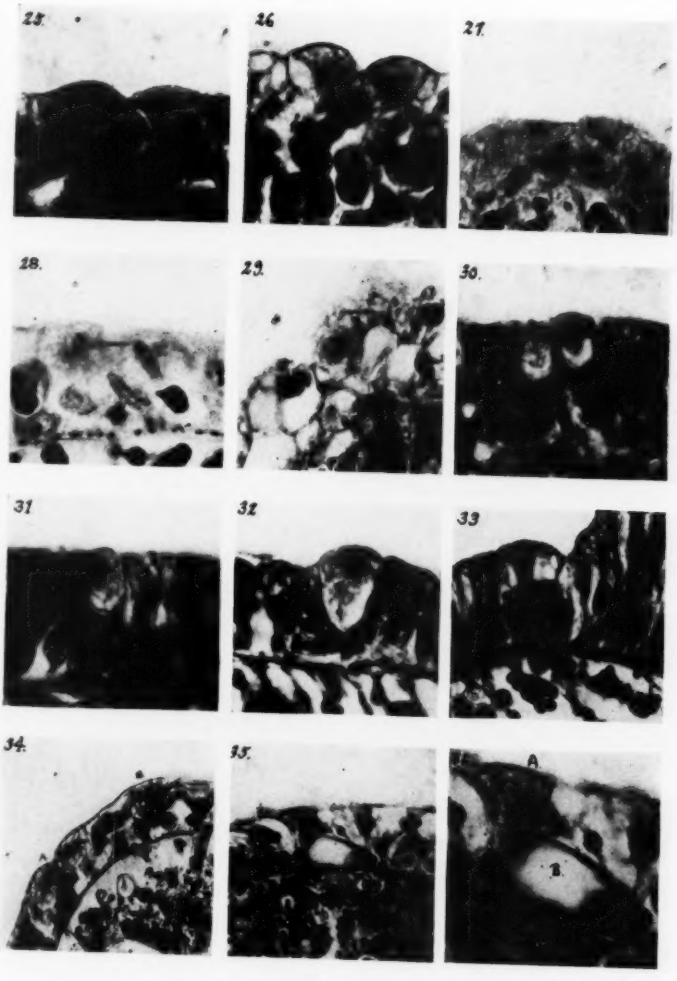
33. The two daughter cells resulting from the division of one of the mucus-secreting cells of the basal disc. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.

34. A cross-section through the lower third of a *Hydra*'s body, showing a telophase at *A*, and a metaphase at *B*. Bouin's; iron-hematoxylin and acid fuchsin. 344 ×.

35. The result of division of an epithelio-muscular cell when the metaphase was not parallel to the mesoglea. Bouin's; iron-hematoxylin. 344 ×.

36. Same as No. 35. The buried cell, *B*, has arisen from the cell *A*. 776 ×.

PLATE III



A close study of one of these preparations (*Hydra* starved 19 days), loaned to me by one of the authors, revealed the following figures given in Table II.

TABLE II

	Prophase	Metaphase	Anaphase	Telophase
Ecto-indifferent	5	8	3	1
Secretory cells	1	1	0	0
Endo-epithelio-muscular	0	0	0	0
Endo-indifferent	0	0	0	0
Ecto-epithelio-muscular	2	1	0	2

We see from Table II then that the division of the cells of *Hydra* may take place under the most adverse circumstances, namely, reduction and resorption, and starvation.

It appears from all the material studied that mitosis is a normal process in the ecto- and endo-epithelio-muscular cells of *Hydra*. It must be admitted that occasionally one finds nuclei which might give the appearance of having arisen by amitosis; but these cases are so rare and so difficult to account for, that they can best be omitted in the present discussion. It may be noted in passing however, that these, which appear to be amitotic divisions, sometimes occur side by side with cells which are going through the process of mitotic division.

If we assumed that the epithelio-muscular cells did not divide but were eventually replaced by cells which were elaborated from the indifferent cells, we should constantly find the indifferent cells of the ectoderm in the rather constant process of enlarging to almost four times their normal size; developing a muscular process with a myoneme; and developing large vacuoles, all of which are characteristic of the ecto-epithelio-muscular cells. If we assumed that the endo-epithelio-muscular cells did not divide we should find the various stages of the endo-interstitial cells in which they are developing muscular processes, food vacuoles, and flagella; but this is not true of any of the material studied so far, for at no time were there found any indifferent cells which might even be mistaken or described as assuming any of the foregoing characteristics of the endo- or ecto-epithelio-muscular cells. It seems then that these endo- and ecto-epithelio-muscular cells are self-propagating by the process of mitosis and that the endo- and ecto-indifferent cells are concerned not with replacing them, but with other processes, namely, with the formation of sperm, eggs, nematocysts, and nerve net cells of the ectoderm; and the formation of nerve cells, secretory cells of the general endoderm, and peristomal gland cells of the endo-peristomal region.

Summary

1. The ecto-epithelio-muscular cells of *Hydra* divide by mitosis.

2. A centriole and asters are present. There are 12 V-shaped chromosomes.

3. These mitotic divisions take place in *Hydra* under all conditions.

4. There is no evidence that the indifferent interstitial cells of the ectoderm or endoderm are elaborated into the large epithelio-muscular cells.

Acknowledgments

I wish to thank Dr. Jovan Hadži of the Zoological Institute, University of Ljubljana, Ljubljana, Jugoslavia; Dr. Max Hartmann, of the Kaiser Wilhelm-Institute, Berlin-Dahlem, Germany; Dr. Fauré-Fremiet of the Collège de France, Paris, France, for their aid and kind criticism while preparing this paper as a National Research Fellow, under their direction.

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MITOSIS IN HYDRA

Mitosis of the Secretory Cells of the Endoderm of Hydra

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Introduction

Schneider (1890) described the secretory cells of the endoderm of *Hydra* as follows: "Die Drusenzellen haben 2 oder 3 Geisseln, sind kurz oval und selten am basalen Ende mit einer Verlängerung versehen. Sie enthalten im Innern ein glänzendes Sekret, das in rundlichen Ballen das Protoplasma erfüllt. Der Kern liegt am basalen Ende. An der Mundscheibe haben sie rundliche Gestalt und zeigen das Protoplasma reducirt auf ein dünnmaschiges Netz. An den Tentakeln fehlen sie. Sie bilden sich aus dem subepithelialen Gewebe." (Page 363.)

Burch (1928), who studied the endodermal flagella, says: "The secreting cells of the general endoderm are club shaped with the smaller end directed toward the mesoglea. The distal end bears one or two flagella. The distal half of the cell is much vacuolated and in well-fed specimens these vacuoles contain darkly staining material called by Schneider, 'Sekretballen.' The basal end is darkly granular and bears no myoneme." (Page 521.)

Downing (1905) maintained that the endodermal cells all divide amitotically; but it has been found that these secretory cells divide by mitosis as well as do the endodermal epithelio-muscular cells. The secretory cells have a mitotic figure slightly similar to the endo-interstitial cells from which they arise in that the astral rays, if present, are only very slightly differentiated, and in the apparent absence of centrosomes. Centrioles are found and lie within the nuclear area.

Material and Methods

Pelmatohydra oligactis Pallas was used in the study of mitosis in these secretory cells. *Hydra* having buds, testes, and ovaries were killed in Bouin's, Zenker's, and Carnoy's. Sections were cut at 5 and 7 microns after embedding in paraffin. Staining was done on the slide with iron-hematoxylin and acid fuchsin. Mountings were made in Damar, and the sections were studied with a compound binocular microscope equipped with 97 × oil immersion objective, and 10 × and 15 × eye-pieces.

Prophase

The early stages of the prophase are difficult to follow. The nuclear membrane remains prominent and stains darkly until the spireme breaks up into chromosomes. The nucleolus also remains, apparently unaltered, up until the disappearance of the nuclear membrane. (See Figs. 2 and 3, Plate I.) The chromosomes are V-shaped and arrange themselves in the equatorial plate with the tips of the V's toward the center of the spindle. The spindle fibers are quite prominent and converge sharply at each pole, which, in many cases, does not touch the surrounding cytoplasm. Astral rays and centrosomes, if present, are not discernible. The centriole up to this stage has not been found, but with the formation of the polar spindle fibers it takes the stain very

Fig. 1

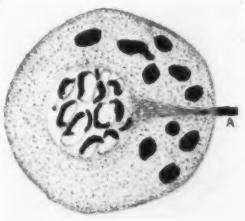

Fig. 2

Text Fig. 1. Section of cell through division center which shows centriole in old nuclear area, *A*.

Text Fig. 2. Daughter cell with remains of spindle fibers at *A;* the centriole being enclosed in nuclear area.

intensely and lies within the old, slightly enlarged nuclear area.

Metaphase

The chromosomes divide longitudinally, division starting at the tips of the V's. As they separate the daughter chromosomes remain connected by interzonal fibers which stain darkly.

Anaphase

As the daughter chromosomes approach the poles their tips fuse upon coming in contact, forming a deeply-staining mass. This mass of chromosomes seems to enlarge slightly when it reaches the area where the new nuclei will be formed. This enlargement is not one of vacuolization, but one of enlargement of the individual chromosome itself prior to vacuolization. The centriole is enclosed within the nuclear area by the formation of the nuclear membrane. (See Text Figs. 1 and 2.)

The interzonal fibers remain until after the cell divides to form the daughter cells. Those portions of it which lie within the area where the new nuclei are to be formed disappear first, leaving a small thread of old interzonal fiber material which remains in the cytoplasm of one of the daughter cells.

Telophase

The polar spindle fibers disappear at the time of the enlargement of the chromosome mass. The chromosomes then fuse and reconstruct the daughter nuclei. The nuclear membranes are re-formed and enclose the centriole within the nuclear area; the nucleolus reappears at about the same time. The old thread of interzonal fibers, which remained in the cytoplasm of one of the daughter cells, disappears at about the time the nucleus reaches its normal size; apparently it is absorbed during the period of growth.

Discussion

Interstitial cells are found both in the endoderm and ectoderm of *Hydra*. As is well known, those of the ectoderm may develop into cnidoblasts, nervous elements, sperm, eggs, and, according to Strelin (1928), they may also be metamorphosed into epithelio-muscular cells

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Plate I

1. Secretory cell from *Pelmatohydra oligactis*. From a preparation fixed in Bouin's and stained with iron-hematoxylin. × 450.

2. Spireme stage of secretory cell with nucleolus still present. Fixed in Bouin's. Stained with iron-hematoxylin and acid fuchsin. × 350.

3. Stage in which the spireme has segmented into chromosomes and the nucleolus has entirely disappeared. Fixed in Carnoy's acetic alcohol and stained with iron-hematoxylin. × 400.

4. Early metaphase seen from the spindle end of the mitotic figure. Fixed in Zenker's. Stained with iron-hematoxylin and acid fuchsin. × 350.

5. Metaphase seen from the side. Fixed in hot Bouin's and stained with iron-hematoxylin. × 350.

6. Late metaphase. Fixed in hot Bouin's. Stained with iron-hematoxylin and acid fuchsin. × 350.

7. Anaphase. Fixed in Zenker's and stained with iron-hematoxylin. × 400.

8. Late anaphase. Fixed in hot Zenker's. Stained with iron-hematoxylin and acid fuchsin. × 350.

9. Telophase. Fixed in Carnoy's acetic alcohol. Stained with iron-hematoxylin and acid fuchsin. × 425.

10. Telophase. Fixed in hot Bouin's and stained with iron-hematoxylin. × 300.

11. Late telophase. Fixed in hot Bouin's and stained with iron-hematoxylin. × 325.

12. Daughter cells. Note old spindle remains in cytoplasm at (*A*). Fixed in Carnoy's acetic alcohol and stained with iron-hematoxylin. × 350.

PLATE I

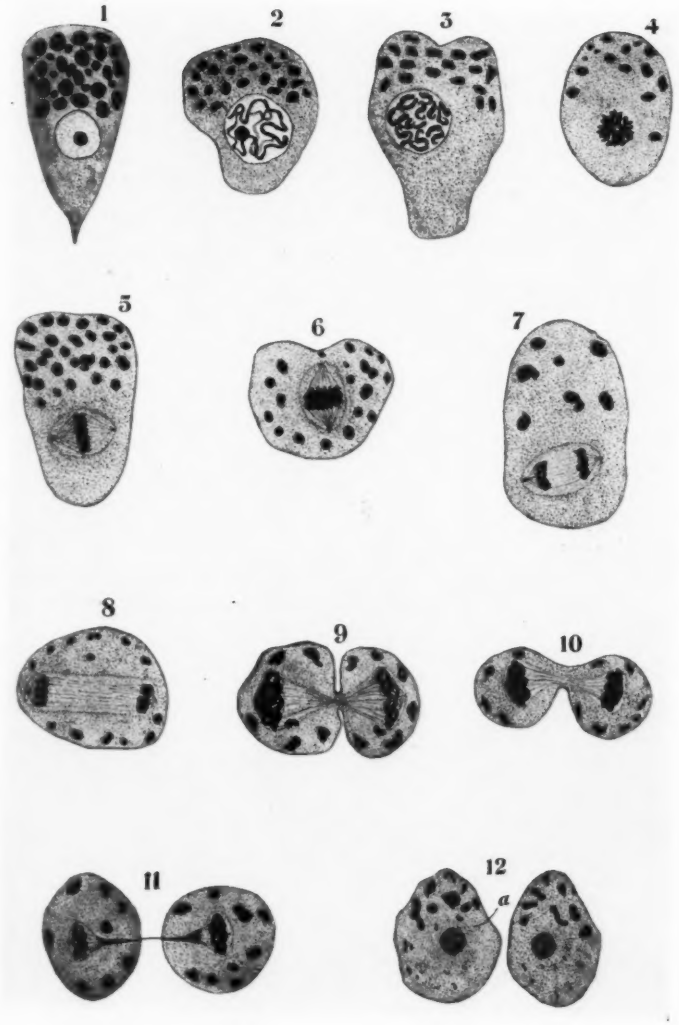
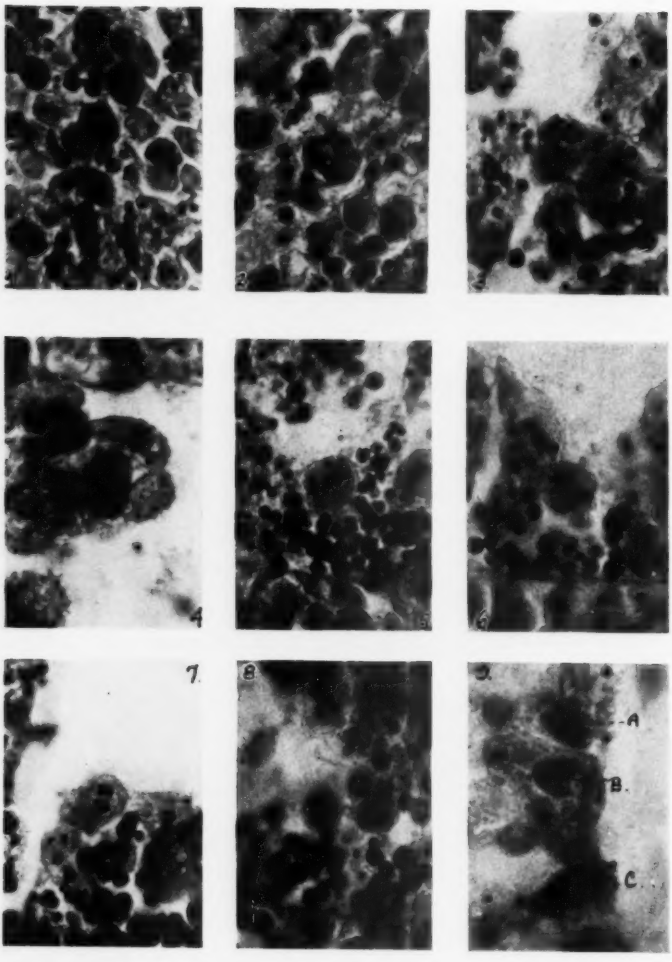


PLATE II



of the ectoderm. The interstitial cells of the endoderm may be elaborated into secretory cells found associated with the epithelio-muscular cells of the lateral walls of the *Hydra's* body. They may also be elaborated into the peristomal-gland cells of the oral region; finally, they may be elaborated into nervous elements associated with the endoderm.

There are, however, several interesting points to be considered in connection with the interstitial cells relative to their location in the *Hydra's* body. Downing (1905), in his work on sperm formation, has shown that those interstitial cells of the ectoderm which are taking part in sperm formation go through a series of mitotic divisions. Again, in his work on ovogenesis (1909) he has shown that the egg arises from an interstitial cell of the ectoderm and, at maturation, casts off two polar bodies. These observations were confined to the interstitial cells which were involved in the sexual activity of the *Hydra;* however, when one studies the interstitial cells of the ectoderm which are developing into cnidoblasts he is forced to the conclusion that after the anlage for a nematocyst has appeared in the cytoplasm of the ectodermal interstitial cell, that cell is incapable of further division.

When one studies the endodermal interstitial cells he finds that they are able to divide by mitosis. When he further studies the cells of the endoderm which arise from these endodermal interstitial cells, he finds that they too are able to increase their number by mitosis after they have been metamorphosed into secretory or peristomal-gland cells; as well as after the secretory particles, which are functionally of a different nature in these two groups of cells, have appeared in their cytoplasm. This is quite in contrast to some of the ectodermal interstitial cells, which, after once being destined to play a part in the general activity of the *Hydra's* life, are incapable of further division.

PLATE II

1. Section through general endoderm to show distribution of the secretory cells. Bouin's; iron-hematoxylin and acid fuchsin. × 776.

2. Early prophase.—Bouin's; iron-hematoxylin and acid fuchsin. × 776.

3. Prophase of a secretory cell which contains already formed secretion granules. Bouin's; iron-hematoxylin and acid fuchsin. × 776.

4. Metaphase of a secretory cell containing secretory granules. Bouin's; iron-hematoxylin and acid fuchsin. × 776.

5. Metaphase seen from the end. Bouin's; iron-hematoxylin and acid fuchsin. × 776.

6. Anaphase. Carnoy's acetic alcohol; iron-hematoxylin. × 776.

7. Anaphase. Carnoy's acetic alcohol; iron-hematoxylin and acid fuchsin. × 776.

8. Late telophase. Carnoy's acetic alcohol; iron-hematoxylin and acid fuchsin. × 776.

9. Daughter cells at *A* and *B*. Compare with normal secretory cell at *G*. Bouin's; iron-hematoxylin and acid fuchsin. × 776.

SUMMARY

1. The secretory cells are able to divide by mitosis.

2. The method of mitosis is similar to that of the endodermal interstitial cells from which they arise.

3. This mitosis may take place after the secretory granules have appeared in the cytoplasm of these cells.

4. The secretory cells are able to increase their number after being destined to play a part in the activity of the *Hydra's* body; in contrast with some of the ectodermal interstitial cells which, after being destined to play a part in the activity of the *Hydra*, are incapable of further increasing their number.

ACKNOWLEDGMENTS

I wish to thank Dr. Jovan Hadzi, Professor of Zoölogy, King Alexander University, Ljubljana, Jugoslavia; and Dr. Fauré-Fremiet, of the Collège de France, Paris, France, for their kind aid and criticism during the preparation of this paper, while working as a National Research Fellow, under their direction.

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THE GROWTH OF THE NUCLEUS IN THE DEVELOPING EGG OF CHLOROHYDRA VIRIDISSIMI

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INTRODUCTION AND HISTORICAL

The development of the egg of *Hydra* has been studied quite thoroughly by many workers, chief of whom are Kleinenberg (1872), Brauer (1891), Downing (1908), Tannreuther (1908), Wager (1909), and Kepner and Looper (1926). All of these workers have pointed out more or less definitely that the eggs arise from indifferent cells, that the developing egg grows at the expense of the surrounding modified interstitial cells which are eventually taken into the egg itself, and that apparently only one nucleus survives and becomes the nucleus of the egg. It is interesting to note in passing that Kepner and Looper (1926) were the first to show that the earlier stages of the development of the egg are more dependent upon the endoderm for their substance than upon the modified interstitial cells of the ectoderm surrounding them. This fact had apparently been overlooked by other workers along this line. Most workers have come to the conclusion that the egg arises from an apparently unmodified, interstitial cell of rather average size, which in its earlier stages cannot be distinguished from the other interstitial cells until the process of ovary formation is very much advanced. Several workers have observed that from one to several eggs may start simultaneous development in an ovary. Usually, however, only one survives and grows to maturity. Downing (1909), on the other hand, found that an ovary of *H. diœcia* may produce several eggs, and further, advances the theory that the interstitial cells which will later develop into eggs are always distinguishable from the other interstitial cells by their size, peculiar nucleus and cytoplasmic inclusion adjacent to the nucleus, and that they are self-propagating and thus handed down from generation to generation.

These studies and differences of opinion, however, deal mostly with the origin of the ovary, the development of the eggs by taking their substance from the endoderm during the earlier stages, and the appropriation of the surrounding modified interstitial cells during the later stages of growth; but since none of these works deal with or demonstrate, ex-

cept in a minor way, the development of the nucleus of the egg, it was decided to make a study of it at its various periods of development.

Material and Methods

Chlorohydra viridissimi was used exclusively in this experiment. A well-balanced laboratory culture was made early in the month of August, and the animals became sexually mature about September first. It was observed that these *Hydra* were producing only one ovary from which apparently only one egg was being matured, so they thus lent themselves quite admirably as material for the study of the development of the nucleus. The method of studying these nuclei was as follows: The *Hydra* were removed to a slide upon which a square of vaseline had been marked out with a match stick. The *Hydra* were placed in the center of this square in as large an amount of Mundie's macerating fluid as possible and then covered with a cover-glass. By drumming very lightly on the cover-glass with a needle it was possible to work the egg out of the ovary; further slight drumming burst the egg and allowed the nucleus to be seen. By carefully sliding the cover-glass to one side or the other it was possible to roll the nucleus out and away from the majority of the surrounding pseudocells where it could be studied, measured, and photographed. Extreme caution was exercised to see that the nuclei were not brought under any pressure so that they remained in their original round condition. When these nuclei were measured, care was taken that the focusing was so adjusted that the nuclei were measured at their equator; this same precaution was exercised when they were photographed. In addition to the above, a series of measurements and observations were made upon nuclei which had been worked out in aquarium water. As far as could be ascertained no measurable shrinkage or swelling could be found, so the work was continued using the Mundie's macerating fluid since the nuclei could be preserved in their almost original condition for from 15 to 20 hours, if precautions were taken to see that the cover-glasses were well sealed with vaseline.

A compound binocular microscope, equipped with 10 ×, 43 ×, and 97 × objectives, and 5 ×, 10 ×, and 15 × oculars, was used in this study.

The Interstitial Cells

Leydig (1848) was the first worker to describe the interstitial cells of *Hydra*. These small cells lie between the epithelio-muscular cells of both the ectoderm and endoderm. They are most numerous in the ectoderm between the epithelio-muscular cells of the upper two-thirds of the *Hydra*'s body; one rarely finds undifferentiated interstitial cells on or in the tentacles: in the stalk and basal disc they are also often en-

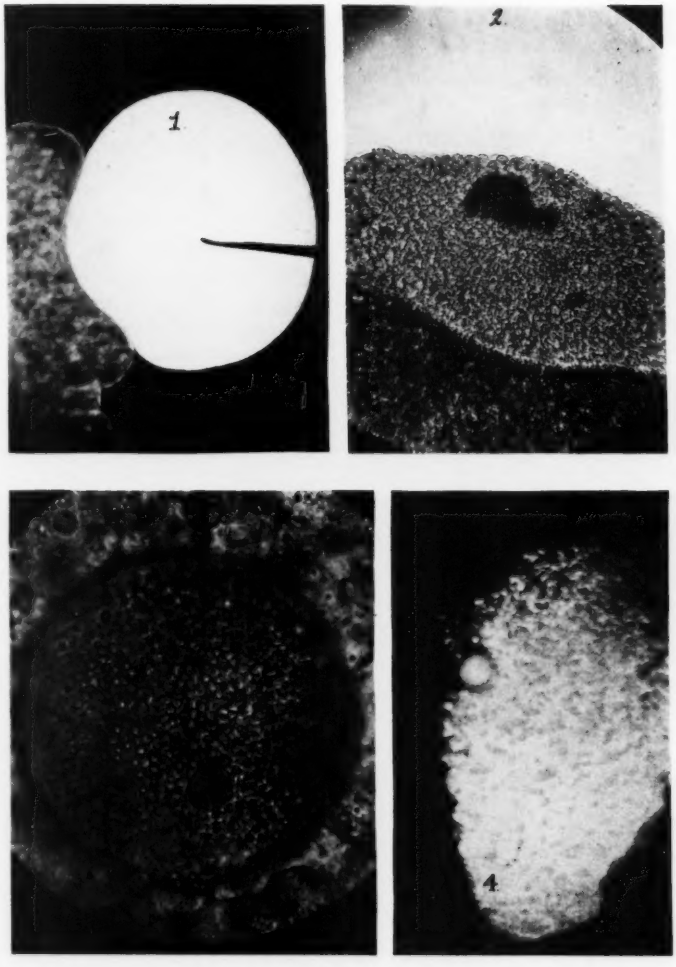

PLATE I

1. Mature egg before process of maturation has set in. Tip of pointer indicates position of nucleus. × 90. Dark field illumination photograph.
2. Dark area indicates a very small egg in the ovary. × 90.
3. Mature nucleus just before process of maturation has set in. × 776.
4. Dark field illumination photograph of an egg crushed in Mundie's macerating fluid. Note size of nucleus as compared to the surrounding pseudocells. × 90.

countered in varying numbers. Various workers have slightly different terminologies in describing these cells, such as follows:

First: Those cells which were developed or were developing into stinging cells have been called "cnidoblasts," "cnidocyts," "nematoblasts," and "nematocyts"; some have even used the word "nematocyst" to designate the interstitial cell with its nucleus, cytoplasm, and cell organelle, or coiled stinging thread.

Second: Some have called all of the cells which lie between the epithelio-muscular cells "interstitial cells" regardless of whether they were in any wise differentiated and,

Third: Some have used the words "indifferent cells" to designate those which were in no wise differentiated. We see then that some ambiguity has arisen through the various terminologies which have been employed. It is true, they are all "interstitial cells," but not all are "indifferent cells." The interstitial cells include all of the cells which lie between the epithelio-muscular cells of the body of the *Hydra* of both the endoderm and the ectoderm. The "indifferent cells," which are also interstitial, are the interstitial cells of both the endoderm and the ectoderm which are not differentiated in any way and which may be modified as needed to various specialized interstitial cells of either endoderm or ectoderm. The total number of "interstitial cells" remains almost constant in relation to the size of the animal; but the number of "indifferent cells" apparently varies greatly at different times; these cells also vary greatly in size and location; sometimes they lie down close to the bases of the epithelio-muscular cells near the mesoglea, at the same time others are found out near the inner surfaces of the epithelium; they may also be found among groups of developing cnidoblasts or in great groups alone. They are occasionally found, apparently unmodified, in developing ovaries and under testes. Their shape varies

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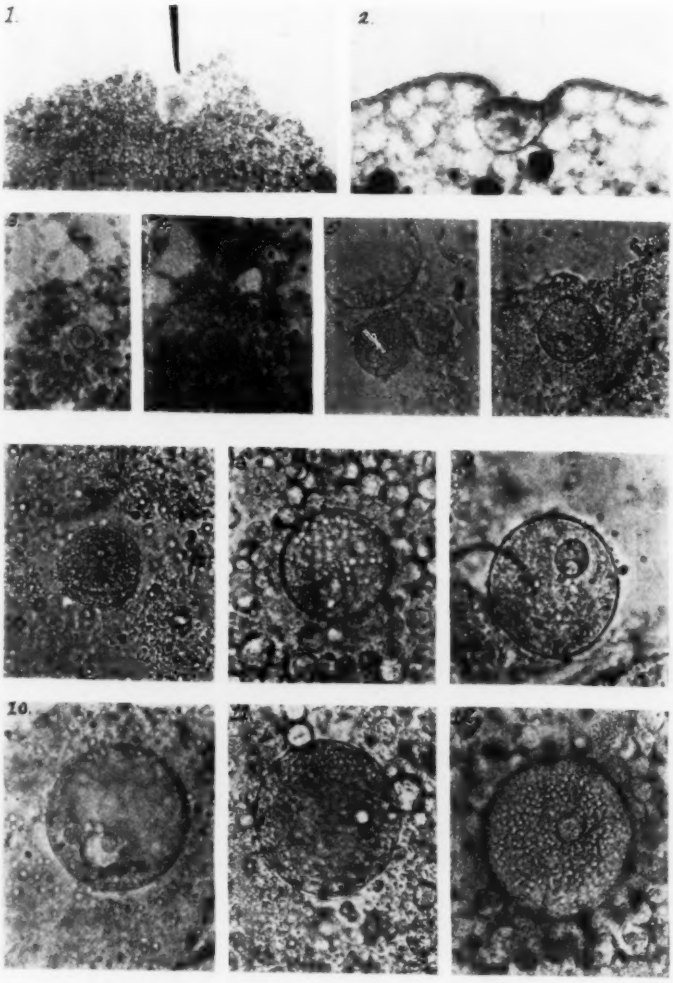
PLATE II

1. Light field illumination photograph of an egg crushed in Mundie's macerating fluid. × 90.
2. The nucleus after maturation as it awaits fertilization at the bottom of a small depression in the egg membrane. Compare with No. 6, Plate III. × 776.
3. Nucleus of a very young egg. Note nucleolus. This was the smallest nucleus found in macerated material. Nuclear diameter 17.5 microns. × 320.
4. From macerated material. Nuclear diameter 24.5 microns. × 320.
5. Macerated material. Nuclear diameter 41.5 microns. × 320.
6. Macerated material. Nuclear diameter 43.80 microns. × 320.
7. Macerated material. Nuclear diameter 54.25 microns. × 320.
8. Macerated material. Nuclear diameter 63.5 microns. × 320.
9. Macerated material. Nuclear diameter 86.75 microns. × 320.
10. Macerated material. Nuclear diameter 94.5 microns. × 320.
11. Macerated material. Nuclear diameter 96.25 microns. × 320.
12. Macerated material. Nuclear diameter 103 microns. × 320.

PLATE II



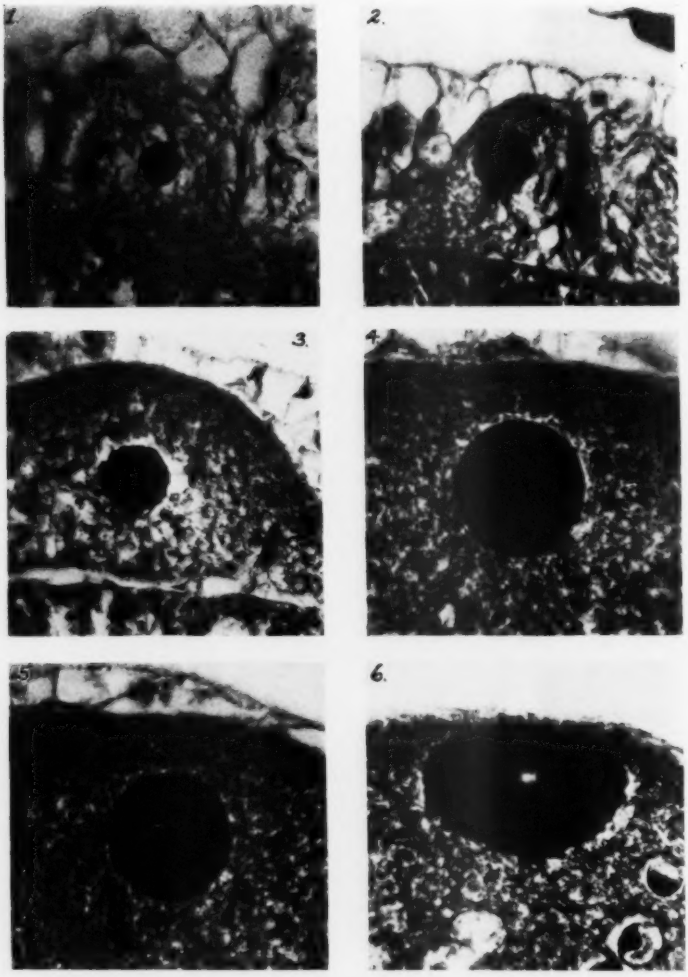


PLATE III

1. Fixed material. Nuclear diameter 9.25 microns. × 450.
2. Fixed material. Nuclear diameter 12.95 microns. × 450.
3. Fixed material. Nuclear diameter 20.50 microns. × 450.
4. Fixed material. Nuclear diameter 38.50 microns. Note how the nucleoli have been increased in 4, 5, and 6. × 450.
5. Fixed material. Nuclear diameter 37.50 microns. × 450.
6. Fixed material. Nuclear diameter 110 × 90 microns. Note that this nucleus is lying just under the egg membrane. × 450.

greatly in accordance with the number of other interstitial cells in the neighborhood and the region of the body in which they are found. The expansion and contraction of the animal's body, it has also been noted, vary the shape of the cells somewhat. During the late prophase of mitosis they assume a rounded condition. The nucleus has a very distinct membrane, and the entire nucleus takes the commoner nuclear stains quite readily and quite intensely. The number of nucleoli varies from one to three, and they vary greatly in their ability to take up various staining solutions.

The Appearance of the Egg and Its Later Development

In sectioned material the egg may be identified much earlier than in macerated material. As has already been pointed out in many works along this line, one of the primary steps of ovary formation is the increase in size of the indifferent cells in the area where the ovary is to be formed. This increase in size continues, in many cases, until these cells have enlarged from eight to ten times their original size. During the process of enlarging, their cytoplasm becomes highly vacuolated and their nuclei degenerated. In studying sections through an ovary one finds that the future eggs do not present any of the characteristics of the modified indifferent cells and take the ordinary stains quite differently from the surrounding cells which they later engulf. When the *Hydra* bearing ovaries are studied slightly compressed the eggs may be identified at an early stage by their color, position in the ovary, and different refraction of the light passing through the ovary. However, it is very difficult to identify the eggs in this manner until they are larger than the average modified indifferent cells which surround them.

Kepner and Looper (1926) have shown that the egg depends first upon the endoderm for its material. This process, as they have demonstrated, is continued until the egg is of quite large size and has developed many pseudopodia which radiate from a more or less central area in all directions between the cells of the ectoderm. Many observations, made on both sectioned and living material, have convinced me that these pseudopodia never invade a region or area in which the indifferent cells are not modified. The boundaries of the ovary are very distinct during all of the process of ovogenesis.

The nucleus grows very fast and apparently is always in proportionate size to the size of the egg. It is very difficult to remove entire eggs from the ovary at an early stage of their development, but during their later stages they may be removed entire if caution is taken. In

this case they are not worked out in Mundie's macerating fluid but in the water from the aquarium. A series of measurements of the eggs and their nuclei is given below in Table I. These measurements were

TABLE I

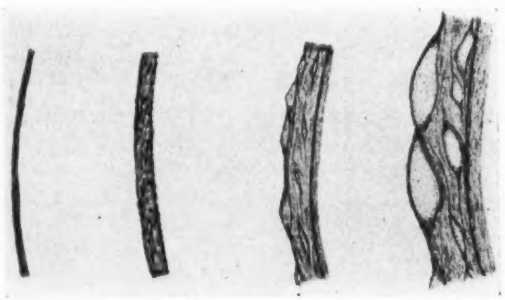
	Egg Diameter *microns*	Nuclear Diameter *microns*
1	1022.17	104.25
2	1035.50	103.5
3	1043.50	101.35
4	1124.2	115.45
5	1145.26	116.49
6	1178.47	116.78
7	1190.55	112.46
8	1232.48	134.19
9	1374.36	146.23
10	1413.57	156.42
Av. diameter egg	1176.004	Av. diameter nucleus 121.712

made on eggs and nuclei just before the process of maturation. We see from this table then that the average relation of diameter size is 1 to 9.66 + for the egg just before the process of maturation took place. Whether this nucleo-cytoplasmic relation has been maintained through the various periods of development is rather difficult to say, but it is interesting to note that several authors have observed that some eggs in their earlier stages are possessed of several nuclei, only one of which survives. Would this indicate then that this cell, which is to be the future egg, can increase in size only in relation to one nucleus or to only a given amount of nuclear material? If we would describe briefly the various stages of development from indifferent cell to the egg as it awaits fertilization, we should see that we have:

1. Indifferent cell (deriving liquid material from endoderm).
2. Very much enlarged indifferent cell (deriving liquid material from endoderm).
3. Very actively amœboid young egg (deriving solid and liquid material from endoderm and ectodermal ovarian cells).
4. The egg ceases amœboid activity and rounds up; nucleus lies near center of egg mass.
5. Migration of nucleus to periphery of egg.
6. Formation of first and second polar bodies with a consequent reduction of nuclear size to about one-eighth of its size at Stage 4.

We see then that this nucleus, up to Stage 4, is controlling a certain amount of cytoplasm; or vice versa, that the cytoplasm is increasing in amount in relation to a certain proportion of nuclear material. It

appears that the cessation of the taking in of more material would indicate that after the nucleus has reached a certain size it controls the action of the cytoplasm, since it has been observed many times that when this amœboid activity ceases and the rounding-up process takes place there are many pseudocells very opportunely placed so that the cytoplasm of the egg could take them in with, may I be permitted to say, very little effort. Would this not indicate that the nucleus is through with its growth, since it allows no more additions to the cytoplasm, and that, at least at this period, it has a definite nucleo-cytoplasmic relation? This growth (to a certain size in relation to the cytoplasm) having been reached, the nucleus goes into the process of maturation during which the nuclear material is reduced. During this process the cytoplasm remains comparatively dormant and does not take any part



TEXT FIG. Nuclear membrane from normal to maturation stage. × 847.

in the cell's activity until the nucleo-cytoplasmic relation has been restored by the entrance of the sperm, which again restores the relation that previously existed.

THE NATURE OF THE GROWING NUCLEUS

By the methods employed in this study, one is unable to see the small dark ovoid body near the nucleus or to measure the relation of the nucleus to the cytoplasm with any accuracy except in the later stages. The nucleus of the young egg, however, can always be distinguished from the nuclei of the surrounding enlarged interstitial cells. This distinction lies in its different refraction of the light and its more solid appearance. The nucleoli vary greatly in number during the growth of the nucleus and are apparently produced by the fusion of the many small nucleoli which are constantly being formed. The larger nucleolus apparently grows also in proportion to a certain nuclear

size. One is constantly impressed, while observing these nuclei, that as far as appearance is concerned from the earliest stages up to the last stages no special change takes place except that of enlargement. The nuclear membrane, which seems to be thicker and more durable than that of other indifferent cells, is quite evident during the entire process of growth. The nodal points of the net enlarge; this growth seems to move inward toward the center of the nucleus during the entire process of growth. At the period of cessation of growth these enlarged nodal points give to the nucleus the appearance of being filled with thousands of large granules all very tightly packed together. The nuclear membrane at this stage is very tough and thin, and one soon learns to distinguish the nuclei which have just completed their growth by this characteristic. Later, this membrane is the first part of the nucleus to show evidences of change incident to the earlier stages of maturation as it increases in thickness and takes on a rather spongy appearance (see text figure). The nuclei which are in the earlier stages of maturation and display membranes such as the above were observed to shrink in size and eventually to become very much wrinkled over their surfaces as if the contained liquids of the nucleus were passing through this membrane into the surrounding medium. Nuclei which displayed the thin tough membranes were observed to maintain the same size over long periods of time, one having been kept for a period of thirty-eight hours.

Summary

1. The nucleus apparently maintains a relation of 1 to 9.66 to the cytoplasm in the development of the eggs of *Chlorohydra viridissimi*.

2. It appears that when the nucleus has attained a certain size no more cytoplasm is added to the egg.

3. During the process of growth of the nucleus the nodal points of the net increase greatly in size.

4. The number of nucleoli increases in relation to the size of the nucleus.

5. The nuclear membrane becomes highly vacuolated just prior to the process of maturation.

6. During the process of maturation the nuclear volume of the egg is reduced to about one-eighth of its volume prior to maturation.

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ION EFFECTS UPON ION PERMEABILITY OF THE *FUNDULUS* CHORION

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I

An electrical potential difference arising between two electrolyte solutions which differ in either concentration or composition is pretty generally interpreted to mean that the net conveyance of positive and of negative ions across the boundary between them is unequal. This is true for the case when the two solutions are free to diffuse into one another. It is also true when they are separated by a membrane which modifies the speeds of ionic diffusion. It is a principle, moreover, equally conceded by those who attribute to the boundary membrane a porous structure permeated by water channels, and by those who assume it to be a strictly non-aqueous phase. Even if water transport across the membrane is occurring simultaneously with ionic diffusion, the magnitude and direction of the observed potential difference must still be a function of the net transport of positive and of negative ions in the two directions. A difference in concentration of positive and of negative ions is admittedly incapable of demonstration by chemical analysis, but this is to be expected; the opposing force of electrostatic attraction must confine the difference to exceedingly minute layers of solution. Absolute rates of ion movement can not be gauged by a measurement of potential difference. But, as a criterion for differential ion transfer, electrical polarity across the boundary between the two electrolyte solutions seems to be remarkably unambiguous.

This criterion has been used in the study of a variety of membranes. In the case of the chorion of single *Fundulus* eggs it seemed to show that at ordinary reactions that membrane is relatively permeable for cations, relatively impermeable for anions (Sumwalt, 1929), a result which satisfactorily explains the long-standing observation of Loeb and Cattell (1915) that K escapes more easily from the egg into a solution of some electrolyte than into distilled water. The same study showed that when the H ion concentration is sufficiently increased, the chorion becomes more permeable for anions than for cations, and that this reversal of preferential ion permeability occurs in KCl solutions at about pH 3.7. It was suggested, however, on the basis of a very limited num-

ber of experiments with two other salts, that the H ion concentration critical for reversal might vary according to the salt in which the membrane potentials are measured.

This suggestion, made originally on the basis of an experimental cue, fits theoretically into a conception of membrane potentials as fundamentally related to diffusion potentials. A study of the reversal point in several salts could therefore furnish evidence for or against the theory that membrane potentials are in some sense modified diffusion potentials.

The problem has also a slightly more practical side. *Fundulus* eggs have been subjected to certain experiments by Loeb (1912–1922) and by Armstrong (1928), which showed a salt antagonism, greater or less according to the salt used, for acid penetration into the egg. Armstrong has shown satisfactorily that this effect takes place chiefly at the ectodermal surface of the embryo. He has not shown, however, that it does not also occur at the surface of the chorion. If a dependence of the ion permeability of the *Fundulus* chorion upon the salt content of the medium, as well as upon the pH, could be demonstrated, it might by analogy furnish an interesting step in explaining the mechanism of salt antagonism for acid penetration through the ectoderm of *Fundulus* embryos.

II

With these two objects in mind, the earlier experiments of Sumwalt on potential differences across the *Fundulus* chorion have been extended to compare three salts, $CaCl_2$, KCl, and K_2SO_4, throughout a range of pH values from 2.0 to 6.0. The particular criterion chosen for this study of ion permeability was the way in which electrical polarity across the membrane was affected by varying the concentration, rather than the composition, of the solutions applied to the membrane. Therefore, the potential differences observed are termed "concentration potentials". Measurements were made in the manner described in detail in the earlier paper. In brief, one measurement of concentration potential in KCl consisted of the following procedures: The chorion of an egg was impaled on the capillary point of a saturated KCl calomel electrode, while the other similar electrode was dipped into the sea water in which the egg was immersed. The sea water was drawn off, KCl M/20 of the desired pH substituted, and a measurement of potential made by means of a simple potentiometric circuit and a sensitive galvanometer used as a null instrument. KCl M/20 was then replaced by KCl M/200 at the same pH, and another measurement made. With this dilute solution, several fresh washings were necessary before the potential attained a

steady value. The algebraic difference between the values in the dilute and concentrated solutions is the concentration potential.

It will be observed from this synopsis that the two solutions of differing concentration were applied successively to the outside of the chorion. It is assumed that the potential difference obtained in this way is equal to that which would be measured if both solutions could have been simultaneously applied outside and inside of the membrane.

Solutions were adjusted to the desired pH values by addition of that acid or base which would add no new ion to the mixture (*e.g.*, KOH or HCl to KCl). A difference of not more than 0.1 of a pH unit, and usually of less than 0.05, was tolerated between the dilute and concentrated members of any given pair of solutions used in a measure-

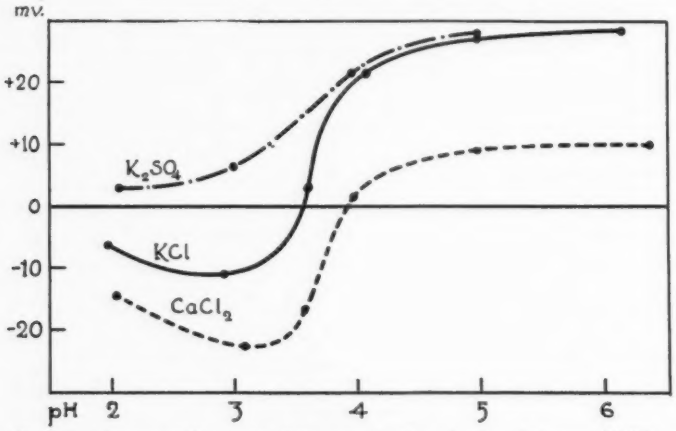

FIG. 1. Concentration potentials across the chorion between M/20 and M/200 solutions at various pH values. The pH plotted is that of the dilute solution, but the pH of the concentrated solution differed from this by less than 0.1. Each point represents an average of values obtained on three different eggs. See Table I.

ment. The necessary pH determinations were made with a quinhydrone electrode calibrated against standard buffer solutions. Those solutions described as having a pH of 5.0 were made up in unboiled distilled water without the addition of any acid or alkali. A few quinhydrone electrode determinations seemed to show that their pH was somewhere between 5.0 and 6.0, as might have been predicted; but determinations of the pH of such solutions are of doubtful meaning, and the exact value of the pH of these more alkaline solutions is not important for the shape of the curves obtained nor for the conclusions drawn from them.

Concentration potentials measured between M/20 and M/200 solutions of KCl at pH 5.0 were of the order of magnitude of 28 millivolts and of such polarity that the dilute solution was positive relative to the concentrated (Fig. 1). In acid solutions, at pH values of 2.0 and 3.0, the dilute solution was relatively negative, and the concentration potential somewhat smaller than in "neutral" solutions. The reversal of membrane polarity occurred along a smooth curve which crossed the line of zero potential at about pH 3.5.

In K_2SO_4 solutions the concentration potentials at pH 4.0 and above were virtually those obtained also with KCl. As acidity increased, the K_2SO_4 curve diverged from KCl, descending with a much more gradual slope toward the zero potential line, and not crossing it at any of the reactions studied in the set of experiments shown (though small reversed concentration potentials at the most acid pH of 2.0 did occur in a few experiments with another lot of eggs).

On the other hand, in $CaCl_2$ solutions, acidity brought about a reversal of concentration potential more readily than in KCl. In the acid-free solutions of $CaCl_2$, concentration potentials were somewhat less than half of those yielded by KCl or K_2SO_4. Reversal of membrane polarity occurred at about pH 3.9, and in acid solutions the concentration potentials were approximately twice those observed with KCl at corresponding reactions.

These experiments were performed on developing eggs ten days old; they had been kept at a temperature of 11° C., however, since their fifth day, so that their development corresponded approximately to that of seven-day eggs. In order to evade difficulties engendered by the variability of *Fundulus* eggs from different batches, the data presented in Fig. 1 were secured within thirty hours on eggs from a single female. Each point in the graph represents an average of concentration potentials from three different eggs. A table of the data from which Fig. 1 was drawn is given to show the small spread of the individual measurements (Table I). Additional preliminary experiments performed on various batches of eggs gave values which differed from these in some respects quantitatively, but not in any qualitative sense. Therefore, although no great significance can be attached to the exact pH value at which the reversals occur, the directional effects of varying degrees of acidity and the comparative influence of the three salts are reasonably certain.

The results, interpreted according to the principle invoked at the beginning of this paper, constitute an answer to the more practical object of this study. They signify that in acid-free solutions the *Fundulus*

chorion is more freely permeable for K^+ than for $SO_4^=$ or Cl^-, and more permeable for Ca^{++} than for Cl^-, whereas in solutions of considerable acidity it becomes more permeable for Cl^- than for any other ion of the salts studied. Furthermore, the tendency of acid to increase the relative permeability of the chorion for anions appears to be augmented by the presence of Ca^{++}, but antagonized by the presence of $SO_4^=$, as compared with an arbitrarily chosen standard behavior in KCl solutions.

TABLE I

Concentration potentials across the chorion measured between M/20 and M/200 solutions. The sign of the dilute solution was positive except where the negative sign occurs. The pH indicated is that of the more dilute solution, from which the concentrated solution differed by not more than 0.1 of a pH unit. Fig. 1 is drawn from these data.

KCl			K_2SO_4			$CaCl_2$		
pH	Concentration Potential	Av.	pH	Concentration Potential	Av.	pH	Concentration Potential	Av.
	mv.	*mv.*		*mv.*	*mv.*		*mv.*	*mv.*
1.98	9.0 – 4.4 – 4.9 –	6.1 –	2.07	3.4 4.4 1.2	3.0	2.04	16.2 – 14.5 – 12.3 –	14.3 –
2.92	7.5 – 13.2 – 12.1 –	10.9 –	3.07	5.0 6.1 9.1	6.7	3.08	24.2 – 23.7 – 19.8 –	22.6 –
3.60	7.2 0.7 1.3	3.1				3.57	17.3 – 15.5 – 16.2 –	16.3 –
4.09	22.0 20.4 21.8	21.4	3.96	19.9 21.1 21.4	20.8	3.98	3.1 1.7 0.2	1.7
5.	28.3 27.5 25.2	27.0	5.	27.1 28.5 28.2	27.9	5.	10.3 8.7 7.7	8.9
6.17	31.1 30.3 23.2	28.2				6.40	6.8 8.8 14.2	9.9

Certain salts differ from one another in their effectiveness as antagonists of acid penetration into *Fundulus* eggs according to the experiments of Loeb (1912–1922), and of Armstrong (1928). The conclusions just described concerning the permeability of the chorion for ions may be brought into relation with those experiments by the following

crude but suggestive theoretical chain. Acid penetration must depend not only on the concentration and speed of the H ion in traversing the membrane, but also upon the concentration and penetrating power of the acid anion outside (or of some cation within the egg capable of exchange for H ions). Hence, increasing acidity in the medium around the egg might favor acid penetration, not only for the primary and obvious reason that it increases the concentrations of the H ion and of the acid anion; but also for the additional reason that it possibly increases the permeability of the membrane for anions. For instance, if the negative charges borne on a membrane were a hindrance to anion penetration, abolition of these charges would bring about increased

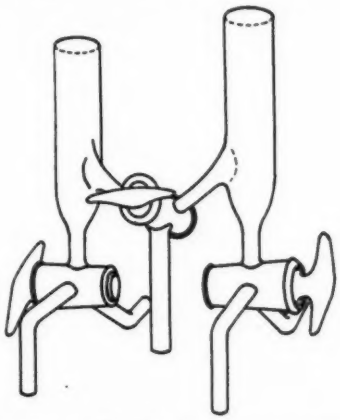

FIG. 2. The apparatus of Dr. Kunitz for measurements of free diffusion potentials. A saturated solution of KCl fills the rear tubes which are to be connected with calomel half cells. In use, the two reservoirs are stoppered so that a difference in level of the two solutions can not cause mixing. If a flowing junction is desired, air may be admitted slowly through these stoppers under the control of screw clips.

penetration of the negative ions. Whether relative increase of permeability for anions signifies an absolute increase of permeability for anions is not revealed by measurements of electromotive force alone, but there is no evidence against such a possibility. This secondary influence of acidity upon acid penetration, then, is the factor which the nature of the salt present could influence, if the salt tends to combine with the proteins of the membrane. A divalent positive ion like Ca may be supposed to be more potent than K in effecting combination with the membrane and so neutralizing its negative charges and bringing it nearer to its isoelectric point. To be specific, acid might be expected, on the basis of the results here recorded, to penetrate more

readily in the presence of $CaCl_2$ than with KCl, and more readily with KCl than with K_2SO_4. It is impossible to make a more detailed application of this suggestion to the work of Loeb and of Armstrong, because neither ever used simultaneously the three salts with which the present experiments have been concerned and because, as has been mentioned already, their experiments dealt with the surface of the embryo rather than with the chorion. The present results must therefore be of interest in a general way, suggestive of a mechanism which might exist at any membrane of structure essentially analogous to that of the chorion. This explanation of the results is purely speculative. But the results themselves, stated in terms of relative ion permeabilities, depend only on the semi-axiomatic premise of the introductory paragraph.

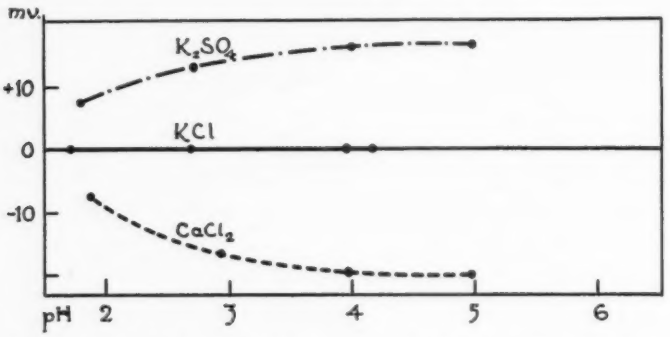

FIG. 3. Concentration potentials across a flowing liquid junction between M/20 and M/200 solutions at different pH values. These results were obtained with the apparatus shown in Fig. 2.

III

To pursue the more fundamental question of the rôle played by diffusion in the origin of membrane concentration potentials, measurements were made of free diffusion potentials between M/20 and M/200 solutions similar to those used in the study of the chorion. They were placed in contact with one another at a flowing liquid junction, and contacts, as before, were saturated KCl calomel electrodes.[1] The results are shown in Fig. 3. Throughout the pH range studied, no potential difference appeared between solutions of KCl. With acid-free K_2SO_4,

[1] I was enabled by the kindness of Dr. Kunitz to use an apparatus which he had designed and used for similar measurements in connection with Loeb's work on anomalous osmosis. A description of it has not been published. It is therefore shown in Fig. 2. The peculiar advantage of this apparatus lies in its prevention of the mixing of the two solutions due to a difference in level in the two cells.

the dilute solution was positive to the concentrated by 16.6 millivolts, an amount which diminished gradually along the descending pH scale to a value of 7.5 millivolts at a pH of 1.8. The dilute solution of $CaCl_2$, on the other hand, was negative to the concentrated by 20 millivolts, a potential difference which likewise continuously decreased with increasing acidity, to reach a value of 7.5 millivolts at pH 1.9.

The diffusion potentials which might have been expected between the solutions used were calculated by the Henderson equation for liquid junction potentials as given by Michaelis (1926, page 179) and are shown in Table II, together with values at corresponding H ion concentrations interpolated on the experimental curves, for comparison. Experimental and calculated values agree very closely in KCl and $CaCl_2$ solutions. In K_2SO_4 an unaccountable systematic discrepancy appears.

TABLE II

Liquid junction potentials between M/20 and M/200 solutions, (*a*) calculated by the Henderson equation, (*b*) obtained by interpolation on experimental curves. The sign of the dilute solution was positive except where the negative sign occurs.

pH	KCl		K_2SO_4		$CaCl_2$	
	Calc.	Exper.	Calc.	Exper.	Calc.	Exper.
	mv.	*mv.*	*mv.*	*mv.*	*mv.*	*mv.*
2.0	0.11−	0.0	7.0	9.0	9.7−	8.8−
3.0	0.27−	0.0	12.3	14.5	17.6−	17.0−
4.0	0.33−	0.0	13.3	16.4	19.5−	19.8−
5.0	0.33−	0.0	13.4	16.6	19.7−	20.0−

Comparative values for concentration potentials across the chorion and across a liquid boundary being now at hand, the question as to what extent these membrane potentials are modified diffusion potentials may be approached. There are two dominant points to emphasize. The first is that when the membrane potentials are "corrected" by the subtraction of the diffusion potentials, the curves for the three salts nearly coincide at the pH value where zero concentration effects are obtained (Fig. 4). This suggests that the membrane is characterized by an isoelectric point, a pH value where the dissociation of acid and basic groups is equal; and that at this pH the membrane influence upon ionic diffusion is withdrawn, leaving ions free to traverse it at the relative rates characteristic of them in free diffusion. The second important point is that at other pH values the membrane exerts an

influence which favors cations in more alkaline solutions, anions in those more acid. The results favor the theory that diffusion potentials do indeed play some part in determining the magnitude and sign of membrane potentials as ordinarily measured, and that at one particular pH value they are the only important factor.

Two minor features of the "corrected" curves (Fig. 4) require brief comment. First, the values of the potentials at pH 2.0 and 3.0 are considerably smaller than at pH 4.0 and 5.0, whereas curves might have been casually expected which were roughly symmetrical with respect to the reversal point. This may possibly be due in part to some obscure electrical property of the membrane. It is certainly partly due to the fact that as the H ion concentration becomes a significant part of the total ion content, the ratio of total electrolyte concentrations

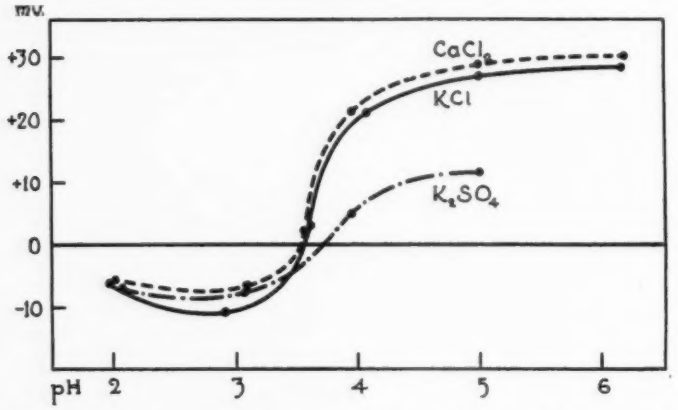

FIG. 4. Membrane potentials minus liquid junction potentials. Such "corrected" membrane potentials probably represent the membrane influence upon concentration effects, more or less freed from the contribution which free diffusion potentials make.

on the two sides, upon which potential difference depends, is somewhat reduced. Free diffusion potentials show a similar diminution with increasing acidity, for a like reason. Finally, it seems quite probable that a destructive effect of the acid may partly abolish the differential ion permeability of the membrane before a measurement of concentration potential can be completed. As was mentioned in the earlier paper, the effects of acid upon the chorion even at pH 3.0 were not reversible.

Second, attention must be called to the fact that certain effects of valence upon the magnitude of concentration potentials across the chorion, predicted and found in the earlier study of the *Fundulus* egg, are found again in the experiments here reported; but they are abolished or inverted when the correction of subtracting diffusion potentials is introduced. Not even a suggestion can be offered, however, in the light of our present knowledge, to explain this relation between valence and "corrected" diffusion potentials. The *Fundulus* chorion is too difficult a material for such measurements to be the basis for extensive theoretical discussion. The results are of interest chiefly for their suggestiveness in connection with concrete problems like that of salt antagonism for acid penetration into *Fundulus* eggs.

Summary

By additional measurements of concentration potential differences across the chorion of single *Fundulus* eggs, certain suggestions made in an earlier paper have been confirmed and amplified. In acid-free solutions of KCl, $CaCl_2$, and K_2SO_4 the chorion was found more freely permeable for positive ions than for negative. Its preferential permeability for cations was reduced in all three salts by increasing acidity. In the first two, it was abolished and then reversed, the pH at which equal permeability for anions and cations was reached being about 3.5 in KCl, and 3.9 in $CaCl_2$. In K_2SO_4, however, reversal did not occur down to pH 2.0.

The results suggest an explanation for the mechanism of salt antagonisms for acid penetration into *Fundulus* eggs, a somewhat practical problem. And, from a theoretical standpoint, they seem to furnish evidence that membrane potentials are fundamentally related to diffusion potentials.

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